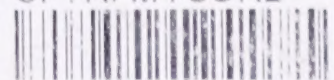


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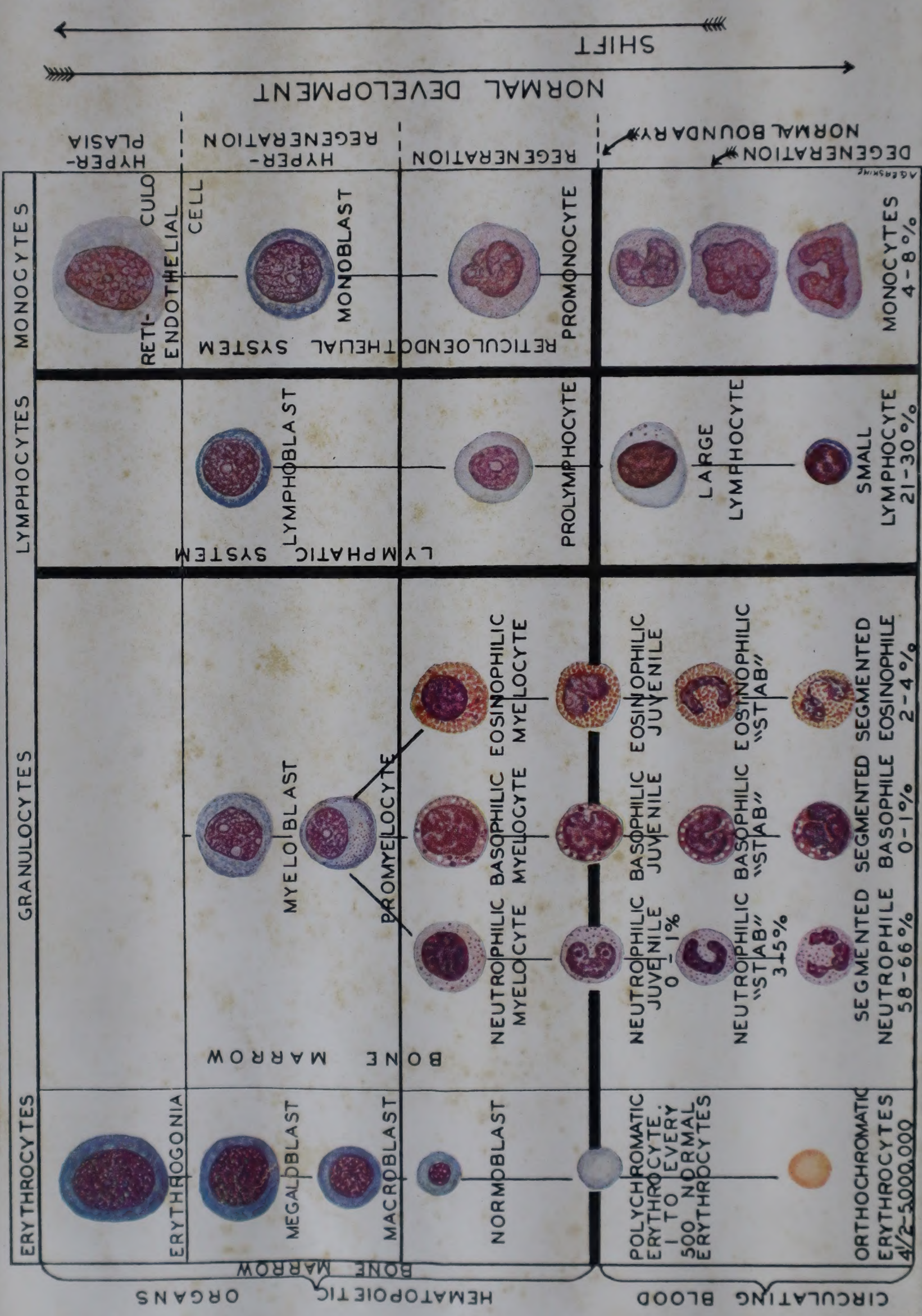
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CLINICAL LABORATORY METHODS AND DIAGNOSIS

VOLUME I

DIAGRAM OF NORMAL DEVELOPMENT OF BLOOD CELLS (SCHILLING) EXPLAINING PATHOLOGIC SHIFTS



CLINICAL LABORATORY METHODS AND DIAGNOSIS

*A Textbook on Laboratory Procedures
With Their Interpretation*

By

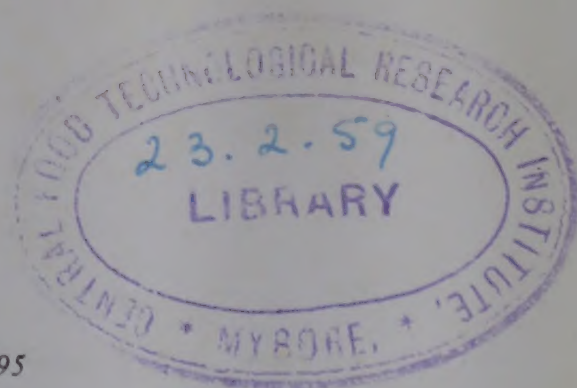
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Reserve, Ret.; Fellow, American
Public Health Association

FIFTH EDITION

VOLUME I

*Text Illustrations, Figures 1-295
Colour Plates I-XXIX*



LONDON

HENRY KIMPTON

1956

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Fourth Edition

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Press of
The C. V. Mosby Company
St. Louis

TO MY WIFE

IDA EMMONS GRADWOHL

THIS BOOK IS AFFECTIONATELY DEDICATED

PREFACE TO FIFTH EDITION

Eight years have passed since the last edition was published. During this time clinical pathology has been enriched with a large amount of new material.

The chapter on Bacteriology has once more been completely revised under the direction of Dr. Oscar Felsenfeld, Professorial Lecturer, University of Illinois College of Medicine, at present Lieutenant Colonel MC, USAR.

In the second chapter, Urine Analysis, many old tests have been eliminated to make room for new methods.

The chapter on Blood Chemistry has been modernized by including methods of converting from visual colorimetry to spectrophotometric analysis; milliequivalents per liter; the new normal blood values for blood chemical constituents. It has been rearranged so that those chemical procedures which are tested for together in clinical diagnosis follow or precede each other; thus, electrolyte balance, the flame photometer, tests for sodium, potassium, chlorides, and CO_2 combining power are placed together. Liver function tests and other function tests are also in one section. The section on liver function tests and liver disease has been revised. It now contains a contribution by Dr. Mitchell A. Spellberg, of Chicago, from his excellent textbook *Diseases of the Liver*, 1954. We wish to express our gratitude to Dr. Spellberg and to Grune & Stratton, his publishers, for permission to quote this material.

Many advances have been made in biochemistry, and great care has been used to make this chapter inclusive of all such advances. The role of potassium and sodium in surgical cases is fully discussed. The Weichselbaum method for proteins in small amounts has been added. This includes the methods for standard plunger type colorimeters and the spectrophotometric methods.

Modern biochemistry is devoting a great deal of attention to the study of body fluids and electrolytes. Dr. J. J. Weinstein, of Washington, D. C., who has been a frequent contributor to this textbook, has prepared a special section covering this subject. Included are CO_2 combining power of blood plasma and its role in acidosis, pH of blood plasma, acid-base balance with the latest methods of detecting changes, particularly as it affects surgical patients. Complete details are given for using the flame photometer with an explanation of electrolyte balance, which it is hoped is so plainly written that it can be easily understood by all who perform or interpret these tests.

Since some laboratories are not yet equipped with a flame photometer, tests for sodium, potassium, and chlorides have been included that do not call for the use of a flame photometer. Especial attention is called to the bedside method of Scribner for the determination of chlorides in plasma, urine, and other fluids, which makes use of a special kit for that purpose.

Included are instructions for preparing a water-chloride balance sheet, important in modern hospital practice in anticipating disturbances in acid-base balance. The method of Lehmann of measuring alkali reserve using small amounts of plasma is also included.

Another important advance is the estimation of protein-bound iodine. These methods are useful for measuring thyroid activity. Complete details for making these determinations have been included in this edition. Major M. C. Hutchinson, of the Fifth Army Laboratory in St. Louis, has given us permission to use the method which was worked out in the Fifth Army Area Medical Laboratory.

The Hematology chapter has been completely revamped. In rearranging and modernizing this chapter, we are greatly indebted to Dr. Harry Agress, Consulting Hematologist to the Jewish Hospital of St. Louis, who has made this a complete text. He has rewritten many of the technics. A section on immunohematology and one on electrophoresis in separating the hemoglobins have been added. The section on blood coagulation is quite new and in accordance with the latest literature. New material has been added throughout the chapter, with many suggestions on technical considerations and modernization of interpretation of blood findings. Dr. Agress has completely revised the bone marrow section.

The chapter on Blood Groups and Transfusions has been completely rewritten, and the latest methods have been added. These methods are largely those used by Dr. Lester J. Unger, of New York City, director of one of the nation's largest blood banks. Dr. Unger has made many important contributions to the subject of blood grouping. During the preparation of this chapter, he graciously consented to have Mrs. Addine G. Erskine, my chief assistant, come to New York and work with him and his staff on the various methods used by him, and to incorporate these facts into this chapter. This was of importance in writing on the general subject of blood groups, especially in relation to blood transfusion. We were fortunate, also, in having the cooperation of Dr. Alexander S. Wiener, who made many valuable suggestions after reading the chapter.

Among the details given in this chapter are new directions concerning titrations of sera for antibodies: anti-A and anti-B, anti-C of the A-B-O system, anti-Rh agglutinins and blocking antibodies, anti-Hr, anti-M, anti-N, and others. At the time of publication of this edition, it can be stated that this chapter is as completely up to date on blood groups and types as it can possibly be made. The method of Unger for using enzyme-treated cells for detection of antibodies in serum, the antiglobulin methods, the conglutinin test, protein media method of cross-matching of Unger are all given completely.

The latest technic for exchange transfusion in erythroblastosis fetalis has been explained. Methods of testing cord and infant's blood for the presence of antibodies derived from the mother are included. An explanation of the test for the C factor of the A-B-O blood group system is also given. The chapter is profusely illustrated with diagrams giving the setup for each test and group of tests. This chapter has been especially designed to give

laboratory workers complete instructions not only in the fundamentals of blood grouping, but also in correct technic which, if lacking, leads to disastrous results. It is our hope that by following correct methods to the letter mistakes in blood grouping may be obviated and therefore future transfusion "accidents" avoided.

Latest methods for securing and preparing whole blood for transfusions are included. There is a section on the heredity of blood groups, and a lengthy table showing genotypes, phenotypes, and children possible in the matings of the Rh-Hr types and subtypes. The chapter closes with a section on the medicolegal considerations of blood transfusions, which should be carefully read and studied by every physician who uses blood transfusions therapeutically. There is also a section devoted to the work of the American Red Cross in blood transfusions.

Some revisions have been made in the chapter on Gastric Analysis. This chapter includes the latest method of tubeless gastric analysis. The discussion of the cytology of gastric secretions is not included in this chapter, but will be found in the chapter on tissue cutting and staining.

A special chapter, Medical Mycology, has been written by Dr. C. W. Emmons, head of the Section on Mycology of the National Institutes of Health, Bethesda, Maryland. Dr. Emmons' outstanding work at that Institute has eminently fitted him for his selection in writing this chapter.

In the chapter on Special Tests, tests for the various estrogens have been brought into line with modern thought. Included in this section is the method for determination of the urinary 17-ketosteroids and a new test for pregnandiol. Some of the older methods have been deleted. The section on suction curettage has been revised by Dr. Robert B. Greenblatt, who wrote the original section. In that part of the chapter devoted to semen appraisal, newer tests have been added, notably the Walker test for phosphatase.

The latest serologic methods are included in the chapter on Serology, and older material has been deleted. Following the suggestions of the Public Health Laboratories of the State of Missouri, certain tests have been omitted: the Eagle test is no longer included since the antigen is not standard, and Dr. Eagle is no longer active in this work. The terminology has been changed to meet the present needs. The original Wassermann test has been left in the book for historical reasons, although we are aware that it is not performed in many laboratories today. We have also left the Hecht-Gradwohl test in the book, not only for historical reasons, but because we still consider it one of the most accurate of the complement fixation tests for syphilis. All the newer methods for performing the VDRL tests have been given; the VDRL test is probably the most accurate of the flocculation tests, and will probably become the standard laboratory test for syphilis. The *Treponema pallidum* immobilization test has been added; this test is used for the clarification of false positive standard serologic tests for syphilis. Complement fixation tests for diseases other than syphilis are given in those sections where such diseases are discussed except that the tests for tuberculosis and gonorrhea are included in this chapter. The serologic tests for syphilis given here are those included in the manual entitled *Serologic Tests for Syphilis*, 1955 Manual, U. S. Department of Health, Education, and Welfare, Public Health Service.

The Parasitology chapter is a condensation of the third volume of the fourth edition. Much of the material of that volume has been abridged so as to include only material needed in the clinical diagnosis of parasitologic diseases, with all references to treatment omitted. I feel that there are so many excellent and extensive textbooks on parasitology that only certain data should be detailed in a chapter in a textbook devoted to laboratory methods. Since Dr. Kourí, who was responsible for most of the third volume in the fourth edition, was unable, on account of pressing duties, to continue the revision, I have revised it and have brought it out as a shorter chapter. The section on spirochetes, viruses, and bacteria has been rewritten and transferred to the bacteriology chapter.

The chapter on Electrocardiography, originally written by Dr. Julius Elson, has been revised by him. A small section is devoted to ballistocardiography.

The term "ml." is the preferred term over the term "c.c." as used throughout this book. This change in the fifth edition has not been made because of the extremely frequent occurrence of the expression "c.c." in standing type.

The index for this revision has once more been completely compiled by Mrs. Addine G. Erskine, chief assistant in all my laboratory enterprises. At this time I wish to acknowledge her help and interest in the development of the book. Without her, this revision could not possibly have been carried out.

To all others who have assisted in the completion of this edition, I wish to express my heartfelt thanks.

R. B. H. G.

St. Louis

PREFACE TO FIRST EDITION

Doctor Victor C. Vaughan said in an editorial published in the October, 1915, issue of *The Journal of Laboratory and Clinical Medicine* that he who practiced medicine without the aid of a laboratory belonged to a past generation of physicians. Granting the truth of this statement then, we know that it is doubly true now.

Thirty years ago laboratory tests were looked upon with only the mildest curiosity; today every hospital, clinic, and physician finds it advisable and necessary to conduct routine and special laboratory tests. Instruction in clinical pathology has become one of the most important branches in the curriculum of the medical school.

This book has been written to help the clinician, the laboratory worker, and the medical student to learn laboratory diagnosis.

The standard technic of accepted procedures is given in strictest detail, together with an interpretation of all data obtained by standard tests. Necessarily the fields of clinical medicine and of surgery have been entered; however no apology is offered for this adventure, since clinical pathology embraces practically every department of medicine.

Believing that the blood is one of the most important fields to be covered in laboratory work, I have stressed the chapter on Hematology, endeavoring to make it reflect the most advanced work on this subject done throughout the world. The Schilling method, clinical application and interpretation, is given in detail. Blood platelets, now thought to be an important factor in causing postoperative emboli and thrombi, are discussed at length.

Sir William Osler said that the place to learn medicine is at the post-mortem table. Physicians and medical students should be encouraged to perform postmortem examinations whenever possible and should learn how to gain the greatest knowledge from each autopsy. For this reason a chapter on Post-mortem Technic has been included.

To the many authors whose textbooks and monographs I have studied and consulted freely in the preparation of this book, I am indebted.

I am grateful to Mrs. Addine G. Erskine for her cooperation. She has made many of the black and white drawings, many of the photomicrographs, and most of the colored illustrations, besides assisting generally in the preparation of the book, including the compilation of the index.

R. B. H. G.

St. Louis

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CLINICAL LABORATORY METHODS AND DIAGNOSIS
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VOLUME I

CHAPTER I

GENERAL CONSIDERATIONS

THE MICROSCOPE

In addition to the compound microscope, it is well to have in the laboratory a simple magnifying glass of six to eight magnifications for the examination of gross parts of organs and plate colonies of bacteria. In using a magnifying glass, hold it close to the eye, and bring it down slowly to the object to be examined. Ordinarily good results are attained at a distance of 25 cm. from the eye.

The Microscope in Detail

The microscope consists of two main parts: the stand and the optical lenses. The stand has two screws for fine adjustment, together with a tube and a stage, usually equipped with a mechanical stage. The lower part of the stand contains a hinge for tipping the instrument and a condenser with an iris diaphragm and a mirror. The stand is equipped with an ocular and objectives. The objectives are screwed into a revolving nosepiece. The tube consists of two parts: the upper part, or the ocular, and the lower part which slips into it and fits into the objective attachment. The distance between the upper edge of the tube and the point at which the objective joins is called the "tube length." It is usually from 160 mm. to 170 mm. in length. It is important to know the tube length because the objectives are corrected for certain lengths. It must be remembered that cover glasses vary in thickness, usually between 0.16 and 0.18 mm. If the cover glass is too thick, the tube length must be shortened, but if too thin, it must be increased by pulling out the tube. This is a point to be considered, especially in the examination of unstained preparations.

The tube is adjusted by means of the coarse and fine adjustments, worked on a rack and pinion. The fine adjustment is so made that one complete turn moves the tube 0.1 mm. Inasmuch as the wheel on the fine adjustment has one hundred notches, the movement of one notch will move the tube 0.001 mm. In general, in using a microscope, bring the tube down cautiously with the coarse adjustment, but to adjust accurately with the fine adjustment, adjust upward, thus preventing undue contact between the objective and the object to be examined. This undue contact will usually injure the objective; it must be carefully avoided.

Microscopes are sometimes equipped with a round, revolving stage which enables one to move the object around. An extra mechanical stage which permits a free movement in two directions gives a method for completely view-

ing an entire slide. This is especially important in making differential blood counts. A mechanical stage purchased with the microscope is well worth the investment. The late models usually have built-in mechanical stages.

The plane mirror should always be used with the condenser except when the source of light is very close to the microscope, in which case the concave mirror is recommended. In general, the light is best adjusted by looking through the barrel of the microscope with the ocular removed and with the image in focus, manipulating the mirror to obtain uniform illumination. For photographic purposes the plane mirror is used almost entirely.

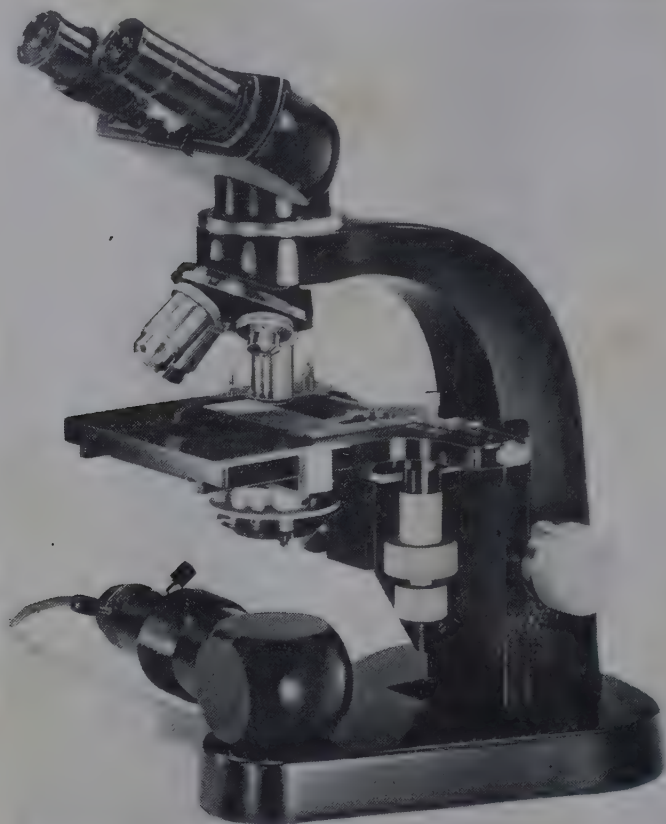


Fig. 1.—Leitz Labolux binocular microscope. (Courtesy E. Leitz, Inc., New York City.)

Below the stage are the Abbé condenser and the iris diaphragm. The condenser is adjusted up and down by means of the rack and pinion. The mirror is fitted below the condenser and is usually plane on one side and concave on the other. The plane side is used with low power magnification and the concave side with high power.

The Optical Explanation of the Path of Light and the Magnification

From below upward, the reflected rays of light from the mirror pass through the condenser, the slide, the cover glass, the objective, and the ocular. The rays of light which are parallel to the condenser converge to one point on the slide, then pass upward through the objective, where they divide, passing up through the tube in almost parallel lines. They are deflected when they enter the ocular, finally converging as they emerge from the upper lens of the ocular and enter the eye as one straight line, giving an image when they strike the retina at separated spaces.

The retinal image is naturally inverted because the rays of light have crossed. The real image formed by the objective lies within the ocular of the

compound microscope; therefore, the objective forms the real image, and our image is looked at, by the eye and a magnifier or ocular, as if it were an object. There are thus two images formed: one by the objective independent of the eye; the other on the retina, by the action of the eye lens of the ocular and the cornea and crystalline lens of the eye. The apparent size of any object depends upon the visual angle. The entire purpose of the microscope is to see objects under a greater visual angle and without this artificial aid.

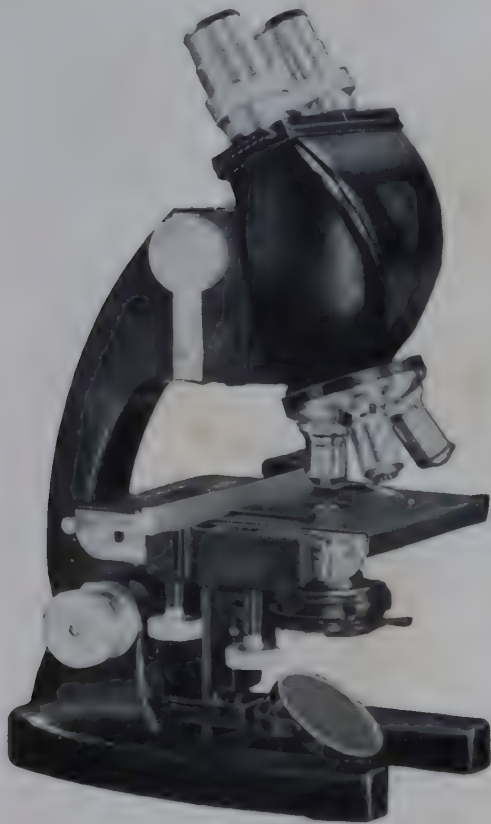


Fig. 2.

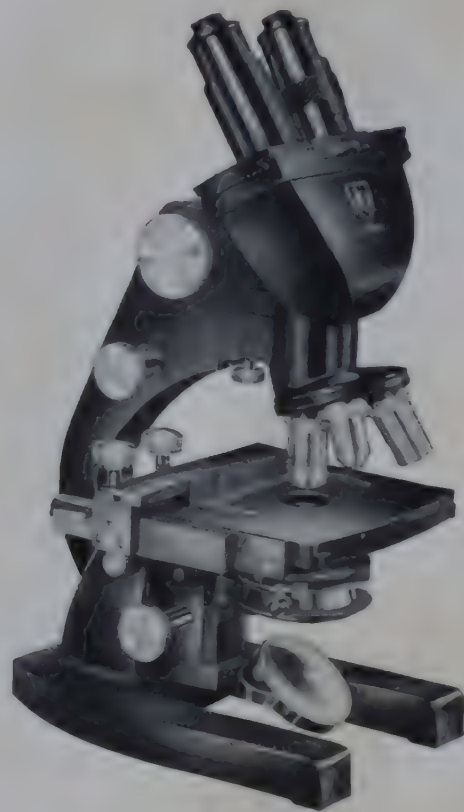


Fig. 3.

Fig. 2.—Spencer binocular microscope, model No. 15 MCH. (Courtesy American Optical Company, Instrument Division, Buffalo, N. Y.)

Fig. 3.—B & L binocular microscope. (Courtesy Bausch and Lomb, Buffalo, N. Y.)

It is well to understand certain technical appellations in optics:

Equivalent Focus.—Objectives are designated by their equivalent focal lengths. This is given in inches or in millimeters. An objective designated as $\frac{1}{12}$ in. or 2 mm. indicates that the objective produces a real image of the same size as is produced by a converging lens whose principal focal distance is $\frac{1}{12}$ inch or 2 mm.

The magnification of the image is approximately the number obtained by dividing the image distance by the equivalent focus of the objective.

For example, if the image distance is 250 mm., the real image of a 2 mm. objective is $250/2$ or 125 times longer than the object.

Designation of Objectives.—American manufacturers designate objectives in inches, Continental manufacturers by letter, usually giving the equivalent focus. The smaller the number or the earlier in the alphabet the letter, the lower the power of the objective.

Parts of the Objective.—(1) Front combination, the part nearest the object; (2) back combination, the part farthest above the object; (3) intermediate or middle combination, between the front and the back lenses.

The Microscope

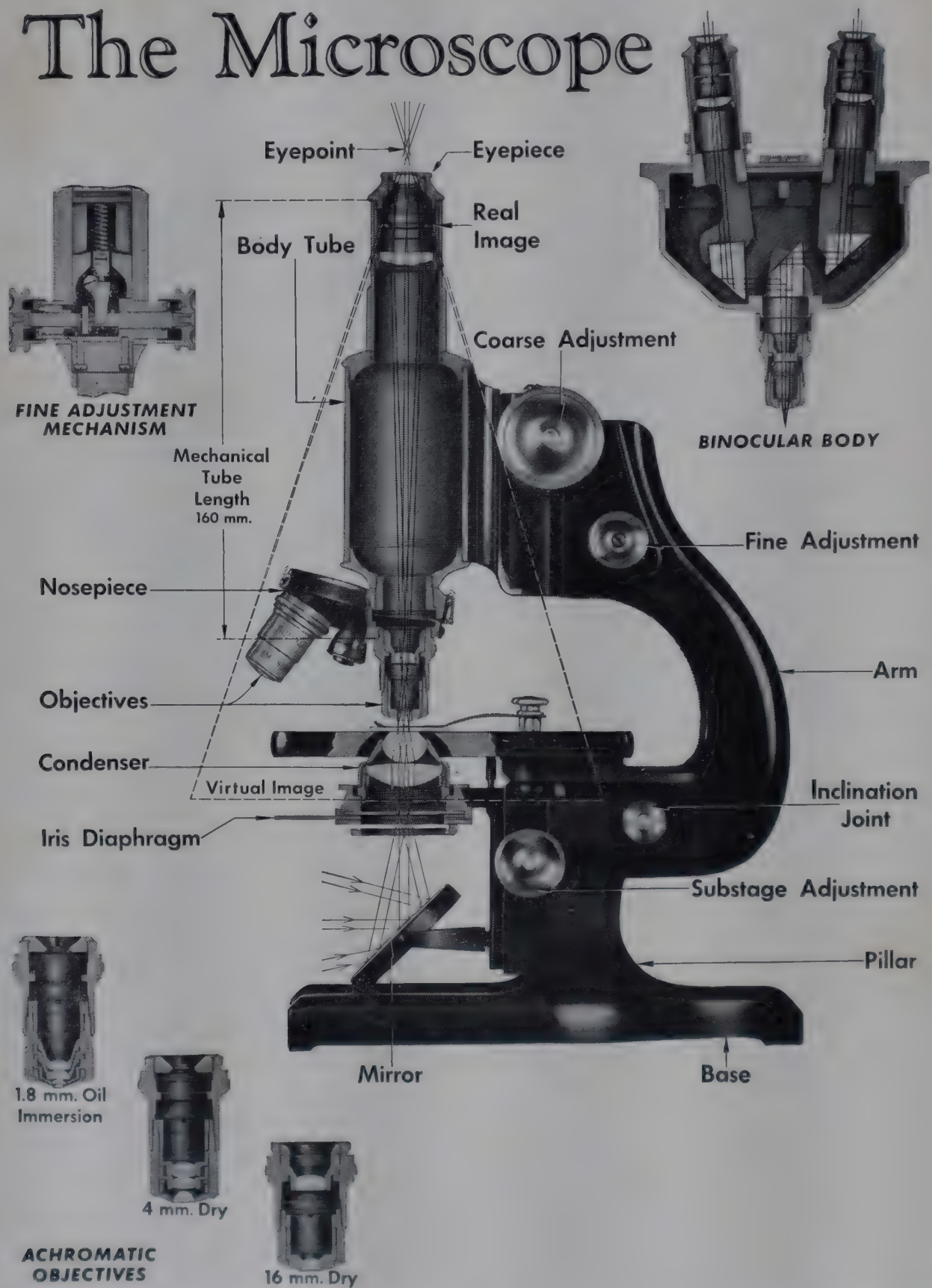


Fig. 4.—The compound microscope showing parts and the path of light through the instrument. (Courtesy American Optical Co., Scientific Instrument Division, Buffalo, N. Y.)

Kinds of Objectives

Dry Objectives.—Only air between the lens and the object.

Immersion Objectives.—There is some type of liquid placed between the lens and the object. The objective is designated by the name of the liquid used; e.g., water immersion, oil immersion, etc. Homogeneous or oil immersion lenses use thick cedarwood oil which has the same refractive index as glass.

Achromatic Objectives are those in which the image is practically free from rainbow colors.

Aplanatic Objectives are those in which spherical distortion is nearly eliminated.

Apochromatic Objectives are those in which, by means of special forms of glass and a mineral fluorite, the color and the spherical corrections have been made perfect by combining rays of three spectral colors into one focus.

Nonadjustable Objectives are those in which the lens system is permanently fixed, while **adjustable objectives** are those in which the distance of the systems of lenses may be changed by the operator.

Variable Objective is a low power objective of 36 to 25 mm. equivalent focus. By means of a collar these combinations may be brought closer together or separated.

Oculars

The ocular is the eyepiece of the microscope. Its main purpose is to act with the eye as a magnifier of the real image formed by the objective. It also helps to correct some of the defects of the objective. Oculars are grouped according to their construction or action:

1. *Positive Oculars* have the real image of the objective formed below all the lenses of the ocular.

2. In *Negative Oculars* the real image formed by the objective is between the lenses.

3. *Huygenian Ocular* is a negative ocular devised by the Dutch astronomer, Huygens, and is the most common ocular used on the microscope. It consists of a plano-convex field-lens and a similar, but higher power, eye-lens, the convex surfaces of both facing downward. Theoretically, the focal length of the field-lens is about three times that of the eye-lens, but in practice the ratio varies with the power, being 1 to 1.5 or 1 to 2 with low powers, and nearer 1 to 3 with high powers.

4. *Compensating Oculars* are either positive or negative oculars chromatically overcorrected to compensate for and correct the residual color defects in the extra-axial portion of the visual field due to the monochromatic front lens of the objectives. They are regularly used with apochromatic objectives, and may be used to advantage with high-angled objectives of the ordinary type.

The Numbering of Oculars is an arbitrary designation. The lower power should be designated by the first letter of the alphabet, or No. 1, and the succeeding letters or numerals should indicate progressive increase in power.

Magnification of Oculars.—It is the custom to mark upon the ocular the increase in magnification it gives to the objective (5x, 10x, etc.). If, for ex-

ample, the real image formed by the objective is 10 times larger than the object, and this real image is magnified 5 times by the ocular, the total magnification of the microscope is 50. If the ocular magnified 10, then the final image would be 100 times the diameter of the object.

The power of the ocular is also indicated by its appearance. A long ocular in which the space between the eye-lens and field-lens is considerable, and the eye-lens relatively large, is usually a low power. If the ocular is short and the eye-lens relatively small, the ocular has a relatively high power.

Use of the Microscope

It is well to put an objective in position and remove it for practice. Elevate the tube of the microscope with the coarse adjustment so that there is plenty of room between its front or lower end and the stage. Grasp the objective lightly near its lower end with two fingers of the left hand, and hold it against the nut at the lower end of the tube or the revolving nosepiece. With two fingers of the right hand take hold of the milled ring near the back or upper end of the objective and screw it into the tube of the microscope or nosepiece. Reverse this operation for removing the objective. This method will prevent one from dropping the objective.

Practice putting an ocular into position and removing it. Elevate the body of the microscope with the coarse adjustment so that the objective will be 2 cm. or more from the object, grasp the ocular by the milled ring next to the eye-lens and the coarse adjustment or the tube of the microscope and gently force the ocular into position. In removing the ocular, reverse the operation. If these precautions are not taken, and the oculars fit snugly, there is danger in inserting them of forcing the tube of the microscope and the objective downward upon the object.

Placing an Object Under the Microscope.—So place an object under the microscope on the stage that it will be in the field of view when the microscope is in focus. With low powers it is not difficult to get an object under the microscope, but the difficulty increases with the magnification and the smallness of the object. It is often necessary to move the object in various directions while looking into the microscope, in order to get it into the field. Time can be saved by putting the object in the center of the field with a low objective before putting the high objective in position.

Field, or Field of View, of a Microscope is the area visible through a microscope when it is in focus. When properly lighted and with no object under the microscope, the field appears as a disc of light. By moving the object within the light circle, different parts are brought successively into the field of view.

In general, the greater the magnification, the smaller is the field. The size of the field is also dependent upon the size of the opening in the ocular diaphragm.

Focusing the Microscope.—The higher the power of the microscope, the nearer together must the object and the objective be brought. To practice

focusing with the low objectives, place a mounted fly's wing under the microscope. Put the 16 mm. objective and the 4x ocular in position. Select the proper opening in the diaphragm and light the object well with transmitted light. Hold the head about level with the stage, look toward the window, and between the object and the front of the objective; with the coarse adjustment, lower the tube until the objective is within about half a centimeter of the object. Look through the microscope and elevate the tube with the coarse adjustment. The image appears dim at first, but becomes very distinct as the tube is raised higher. If the tube is raised too high, the image becomes indistinct, and finally disappears. It again appears if the tube is lowered the proper distance.

The Mirror.—When the microscope is well focused, try both the concave and the plane mirror and note the effect. Pull out the draw-tube from 4 to 6 cm., thus lengthening the body of the microscope and making it necessary to lower the tube. Push in the draw-tube very cautiously to avoid forcing the objective against the object. All microscopes are not equipped with draw-tubes.

In general, it is well to move the preparation while focusing. Always focus up; never down.

Working Distance.—By working distance is meant the space between the simple microscope and the object, or between the front lens of the compound microscope and the object, when the microscope is in focus. This distance is always less than the focal length of the objective. Since the working distance of an objective is always less than its equivalent focus, be sure to use cover glasses thin enough so that specimens of any thickness can be used.

In working with oil immersion objectives, place a drop of special oil for immersion purposes over the cover glass, bring the objective slowly down into the oil by means of the coarse adjustment, holding the end about level with the stage of the microscope until the objective is well near the surface of the cover glass but not touching. Then look through the microscope and slowly focus.*

Illumination for Microscopic Work.—North daylight is best and is the most uniform, especially when the sky is covered with white clouds. Since it is not always possible to have north light, reflected light must very frequently be used. The best method of artificial illumination is that obtained by placing an electric bulb behind a Florence flask containing distilled water with a pinch of copper sulphate. This gives an illumination similar to daylight. Special microscope lights are available.

Position of the Condenser.—The condenser should be so adjusted that the maximum illumination is obtained in stained specimens using an oil immersion objective. When using unstained material, the condenser should be lowered or may be removed entirely, and the mirror so adjusted that only a dim light is reflected upon the object. Some of the light can be shut off with the iris diaphragm when examining unstained material.

*South of the equator, use south light.

It is well to remember that the direct rays of the sun should never be reflected into the eye of the observer; this will cause injury to one's vision.

Care of the Microscope.—The microscope should be kept clean and should be handled very carefully to avoid breakage. It is important to handle oculars and objectives in such manner that they will not fall and be broken. After using, the microscope should be cleaned and covered with a bell jar or replaced in the box which is usually furnished with it. No liquids of any kind should be placed upon the microscope, especially acids, alkalies, alcohol, and chloroform. These chemicals remove the lacquer from the microscope and will dissolve the cement which holds the lenses in position.

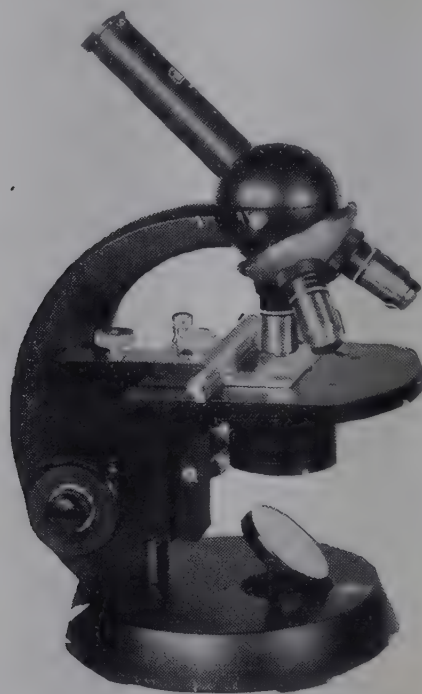


Fig. 5.—Zeiss monocular microscope, model KFM3. (Courtesy Graf-Apsco Company, Chicago, Ill.)

To clean the mechanical parts, place a small quantity of olive oil on a piece of gauze or lens paper and rub well. Then wipe off the oil with a clean dry chamois or lens paper. The optical parts require absolute cleanliness. The glass surface should never be touched with the fingers, because this leaves fat upon the surface and makes it difficult to clean. Where the objectives are left in position, be sure that the revolving nosepiece is closed and an ocular is left in the upper end of the tube to prevent dust from falling down upon the back lens of the objective. One of the best means to detect anything wrong with the objective is to examine the eye-point with a magnifier. The field should be lighted well and the aperture filled about two-thirds full of light. If there are any defects or smears of balsam or liquids on the front lens, unsealing of the combinations, or dust on the upper face of the back lens, the defect can be seen in the eye-point.

Another and very certain method of detecting imperfections is to rotate the different elements while looking into the microscope. If the defect is in the mirror, it will change in position when the mirror is moved. This is true of all the other elements. Defects in the ocular are strikingly shown by rotating it.

It is well to have on hand an ample supply of good grade lens paper. Once used, it can be thrown away. If it contains no dust or sand, its bibulous character is sufficient to aid in removing liquid or semiliquid substances. Dust may be removed with a camel's hair brush, or with the lens paper. Cloudiness may be removed from the glass surfaces by breathing on them and then wiping them quickly with a soft cloth or lens paper. Cloudiness on the inner surfaces of the ocular lenses may be removed by unscrewing and wiping them. A high power objective should never be taken apart by an inexperienced person. Water may be removed with a soft cloth or lens paper. Glycerin may be removed with cloth or lens paper saturated with distilled water. If blood or other albuminous material is dried on the glass, it may be removed readily by adding a very small quantity of ammonia to the water (1 c.c. of ammonia to 100 c.c. of water). Mallinckrodt Lens and Process Screen Cleaner (5801)* is excellent for cleaning oculars and for removing dry blood from objectives. Do not use it on the oil immersion objective.

Canada balsam, or other oily substances, may be removed by lens paper moistened with xylol. This must be done as rapidly as possible, so that none of the liquid will have time to soften the setting of the lenses.

Care of the Eyes.—Keep both eyes open, using one eye for a while, then the other. The binocular microscope is advantageous in that one uses both eyes all the time. In the beginning, do not use a microscope over too long a period, and, in general, do not work with a microscope after the eyes have become fatigued.

Position and Character of the Worktable.—The worktable should be very firm and large, and should be at the so-called desk height. There should be an adjustable stool, preferably with a back rest.

Test of the Microscope.—While the manufacturers are all very conscientious in sending out only good instruments, it sometimes happens that a second-hand instrument comes before us for purchase. There are certain fundamental points that even beginners may learn in regard to testing. Expert testing, however, requires a world of optical experience. The fundamentals for a fairly good working instrument are:

Mechanical parts should be firm. Bearings should work smoothly. The mirror should not wobble, but should remain in any position in which it is placed. The coarse and fine adjustments should work perfectly. The coarse adjustment may be too hard or too easy to work. If too hard to work, the bearings of the pinion are too tight or the gliding surfaces too sticky and not properly lubricated. If the bearings are too tight, loosen the screws very slightly; if the bearings are not lubricated, wet a cloth with lubricating oil and rub the gliding surfaces. If the tube runs too easily, the bearings of the pinions are too loose and the screws must be tightened.

The fine adjustment should be so well arranged that there is no motion except in the direction of the optical axis. If this fine adjustment is not in order, there will be swaying of the image with central light. If the fine adjustment gets out of order, send the instrument to the maker for repair.

*Mallinckrodt Chemical Company, St. Louis, Mo.

Markers.—It is advantageous to have a marker on the ocular to point out definite places on the microscopic object. This marker can be made as follows: remove the eye-lens from the ocular and with a little Canada balsam fasten a camel's hair to the upper surface of the ocular diaphragm so that it will project halfway across the opening. Most of the microscope manufacturers now make a microscope slide marker which fits on the nosepiece like an objective and has a very fine diamond point which is excentric. After the object to be marked is found, the objective is swung out and the object marker is swung into position, adjusted with the fine adjustment down so that the diamond point touches the slide firmly. With a screw movement the objective is made to turn one complete revolution which puts a circle into the glass slide by virtue of the excentric position of the diamond point.

The Dark-Field Apparatus is a part of optical apparatus that was introduced shortly after the discovery of the *Treponema pallidum* by Fritz Schaudinn. It is especially important for demonstration of this microorganism, and is also useful in studying specific granulations of blood cells. It has been erroneously called the ultramicroscope. It is not an ultramicroscope. Dark-field apparatus are made by all optical manufacturers. They are not interchangeable. A Spencer dark-field fits only a Spencer microscope, a Zeiss a Zeiss microscope, etc. Microscope manufacturers now make an adapter which permits their dark-field apparatus to be used on any microscope. The purchaser must specify the type of microscope when buying the adapter.

The dark-field apparatus consists of a stop or funnel, which fits into the oil immersion lens; a special condenser with a dark spot on its upper surface; and a very good source of illumination, a carbon arc lamp or a high-powered Mazda bulb. The later models have a small lamp which fits into the condenser, with a resistance between the street current and the lamp to reduce the voltage. The newest Zeiss dark-field employs a special oil immersion objective without a stop or funnel and a special adjustable condenser, utilizing a light of considerable intensity.

The principle of the apparatus: the numerical aperture of the objective must be low; that is, less than 1.00. Since the oil immersion objective has an aperture of between 1.20 and 1.40, we employ the funnel stop to reduce this to a point less than 1.00. With this apparatus, the object is light and the background dark. This is due to the fact that the object is illuminated by a beam of light which cannot get into the objective either because the rays are too oblique or because they are cut off before reaching the eye; in either case the light on the background never reaches the eye. Only that which is refracted, reflected, or diffracted by the object reaches the eye. Consequently the appearance is that of a bright object on a dark background. The purpose of placing a drop of immersion oil between the condenser and the lower surface of the slide may be explained as follows: Great obliquity of light is used in high-power work. If the light in the condenser is above 41 degrees (the critical angle), it will not emerge from the terminal face of the condenser, but will be totally reflected. By using the oil on the condenser, the light passes on directly to the object without change in direction.

To use the dark-field, prepare the specimen properly. Have a very thin preparation, use a perfectly clean glass slide, free from scratches, and a particularly thin cover glass. In attempting to find *Treponema pallidum*, it is important to follow certain very definite directions:

A very ingenious apparatus for obtaining serum for dark-field examination for *Treponema pallidum* has been devised by Becton-Dickinson Co. It consists of a sterilizable glass cup with a strong rubber suction bulb.

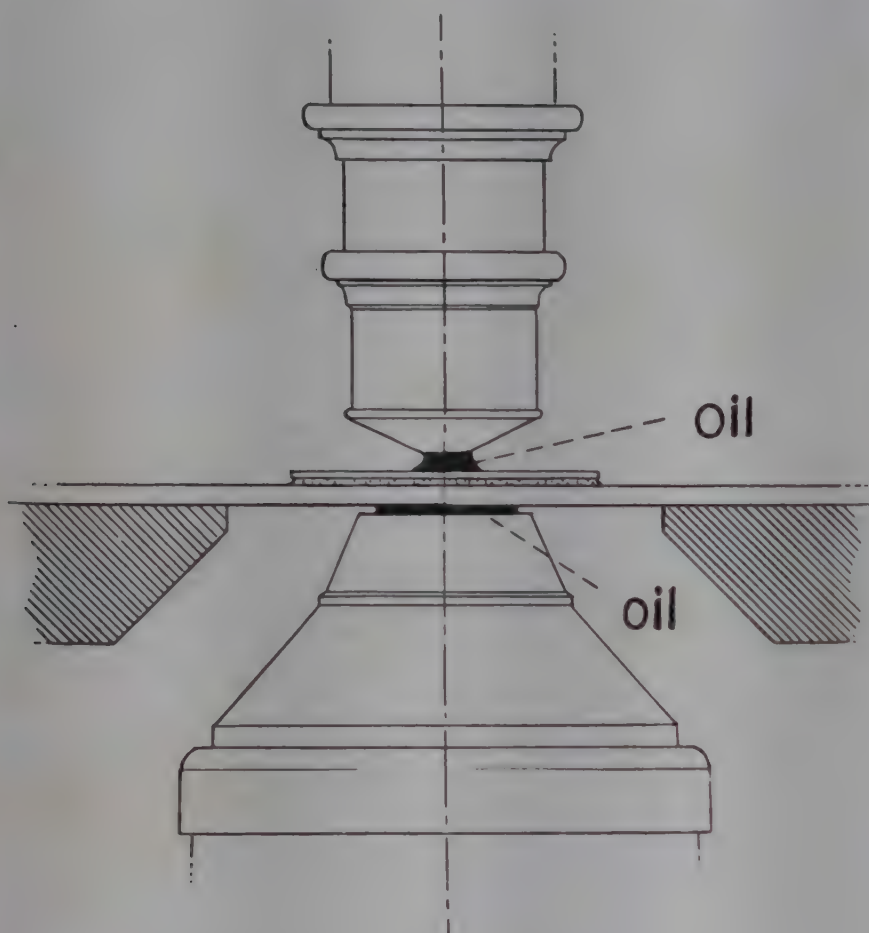


Fig. 6.

Fig. 6.—Diagram of dark-field illumination.



Fig. 7.

Fig. 7.—Suction bulb for dark-field work. (Courtesy Becton-Dickinson Co.)

A very good method for obtaining fluid by suction is that described by Rosenthal.¹ This apparatus, which can be readily made, consists of a 2 c.c. syringe barrel, a 10 c.c. syringe (barrel with plunger), a length of rubber tubing approximately 6 inches long (thick walled), a small piece of glass tubing, and a small piece of cotton attached as in Fig. 8.

The advantages of this method are: (1) materials are readily available; (2) apparatus is easily manipulated; (3) a clear fluid free of detritus plus the amount obtained facilitate dark-field examination; (4) the apparatus may be readily cleaned and sterilized; and (5) the technician is safe from secondary infection.

1. The operator should wear sterile rubber gloves.

2. The surface of the lesion should be cleaned with sterile saline so as to remove most of the extraneous bacteria, care being taken, of course, to avoid hemorrhage. Hemorrhage will introduce into the preparation too many red

¹Hospital Corps Quarterly, U.S.N., Jan., 1946; Lab. Digest 9: 9, Feb., 1946.

corpuscles which will defeat the very object of the examination by hiding the spirochetes in the dark-field. While syphilitic primary lesions are extremely contagious, it is a well-known fact that the spirochetes are not easily found on the superficial parts of the lesion. It is, therefore, necessary in order to make a successful dark-field examination to get deep tissue fluid from these lesions. This is accomplished in several ways, preferably by the use of suction. (See Figs. 7 and 8.) Before applying suction, it is also advantageous to place a drop of xylol on the lesion; this acts in a measure as a counterirritant and brings serum to the surface. The ideal material for the examination is a clear, straw-colored serum free from red blood cells. A drop or two of this serum is aspirated from the surface of the lesion with a fine capillary pipette, and then ejected upon the surface of the slide. This is immediately covered with a thin cover glass, and pressure is brought with the finger on the cover glass so as to produce a uniform, thin medium between cover glass and slide. Attention once more is called to the fact that this material is dangerous; the finger pressure should be carried out with a clean rubber glove.

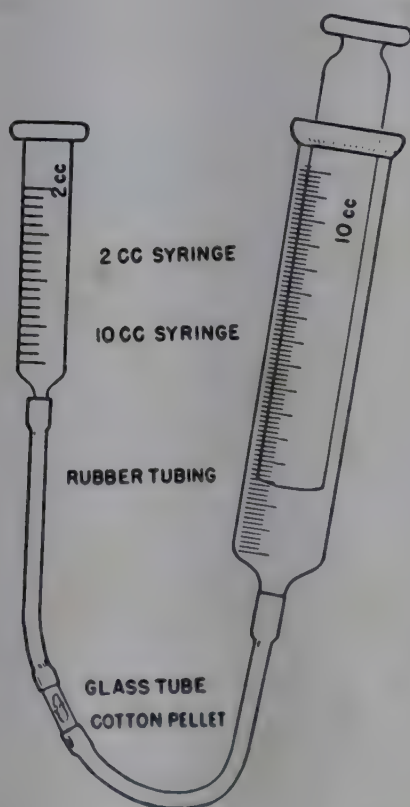


Fig. 8.—Apparatus designed by Rosenthal for obtaining fluid by suction for dark-field examination.

Now, having obtained the specimen properly, prepare the microscope. First, place a funnel in the oil immersion lens. Next, place the condenser with a dark spot and prepare the illumination for the microscope. Next, swing the low-power objective into position. Now look through this objective in order to find the dark spot on the condenser which is brought into focus. This dark spot must be exactly centered; that is, the two concentric circles must just encircle the edge of the dark spot as seen through the low-power objective. Having centered this, bring the condenser up to the stage of the microscope, place a drop of immersion oil on the condenser, place the slide on top of this, and make such pressure that the drop of oil will be uniformly distributed between slide and condenser; place a drop of oil in the usual manner on top of

the cover glass, bring the oil immersion lens down to this drop of oil, and then focus carefully. Living objects are seen as brightly illuminated spots with a dark background. Proper observation, if the lesion is a true syphilitic one, will reveal the spiral-shaped, rapidly moving spirochetes.

With the use of the type of dark-field apparatus such as the Zeiss Kardiod condenser, it is not necessary to center as above described. A special objective is used with no funnel. This is a more satisfactory method. The object is brought into view by simply moving the lever at the lower side of the condenser.

In addition to the examination for spirochetes, the dark-field is useful for certain studies of blood specimens. To study blood in this manner, take up a small, very fresh drop of blood on a large, clean cover glass, selecting, of course, a thin cover glass. Superimpose this on a clean slide so that the blood spreads out spontaneously. The blood must just reach the edge of the cover glass. For prolonged observation, frame with petrolatum. We see erythrocytes with their rouleaux formation; blood platelets; lymphocytes as almost homogeneous globules with darker nuclear spaces; granulocytes as follows: neutrophils with fine granulations, eosinophiles with coarse granulations and somewhat yellow. The nuclei of these cells appear as vacuoles. The monocytes show a fine, dusty cytoplasm. Upon heating, ameboid motion of all leukocytes takes place, with granular ciliation. Lymphocytes and monocytes are sluggish in their motion. After three to ten minutes we see fibrin needle formation, with motile debris and derivatives. The very small "powdery" particles of blood (hemokoniae), which become immediately visible in the dark-field, are mainly fat particles. They are much increased after meals. It is claimed that they constitute a clinical control of fat resorption and of the working capacity of the reticuloendothelium. Lack of hemokoniae one to two hours after a fatty meal indicates disturbed function of gall bladder or pancreas; their prolonged presence, a pathologic retention in the reticulum.

The dark-field is of special advantage, also, for large malarial parasites. They may be identified immediately by their pigmentation and lively granular circulation, and their ameboid motion; as for trypanosomes, they can be identified as easily as the spirochetes already described.

Fluorescent Microscopy.—Refer to Chapter X, Bacteriology, Vol. II.

Phase Microscopy¹

Phase microscopy is a departure from the older methods of microscopy. The phase microscope reveals detail in transparent materials having regions of slightly differing absorption or with different indices of refraction provided the detail is not too small for the resolving power of the objective used. Such details often are invisible with technics previously available as brightfield, dark-field, polarization, etc. Colored, transparent materials may be examined, and the additional contrast from color fibers may be combined with the increased visibility from the use of diffraction plates.

Bennett, Jupnik, Osterberg, and Richards² mentioned the medical possibilities in physiology, parasitology, pharmacology, identification of microorgan-

¹Lab. Digest 10: 7, 1946.

²Transactions of the American Microscopical Society 45: 2, 1946.

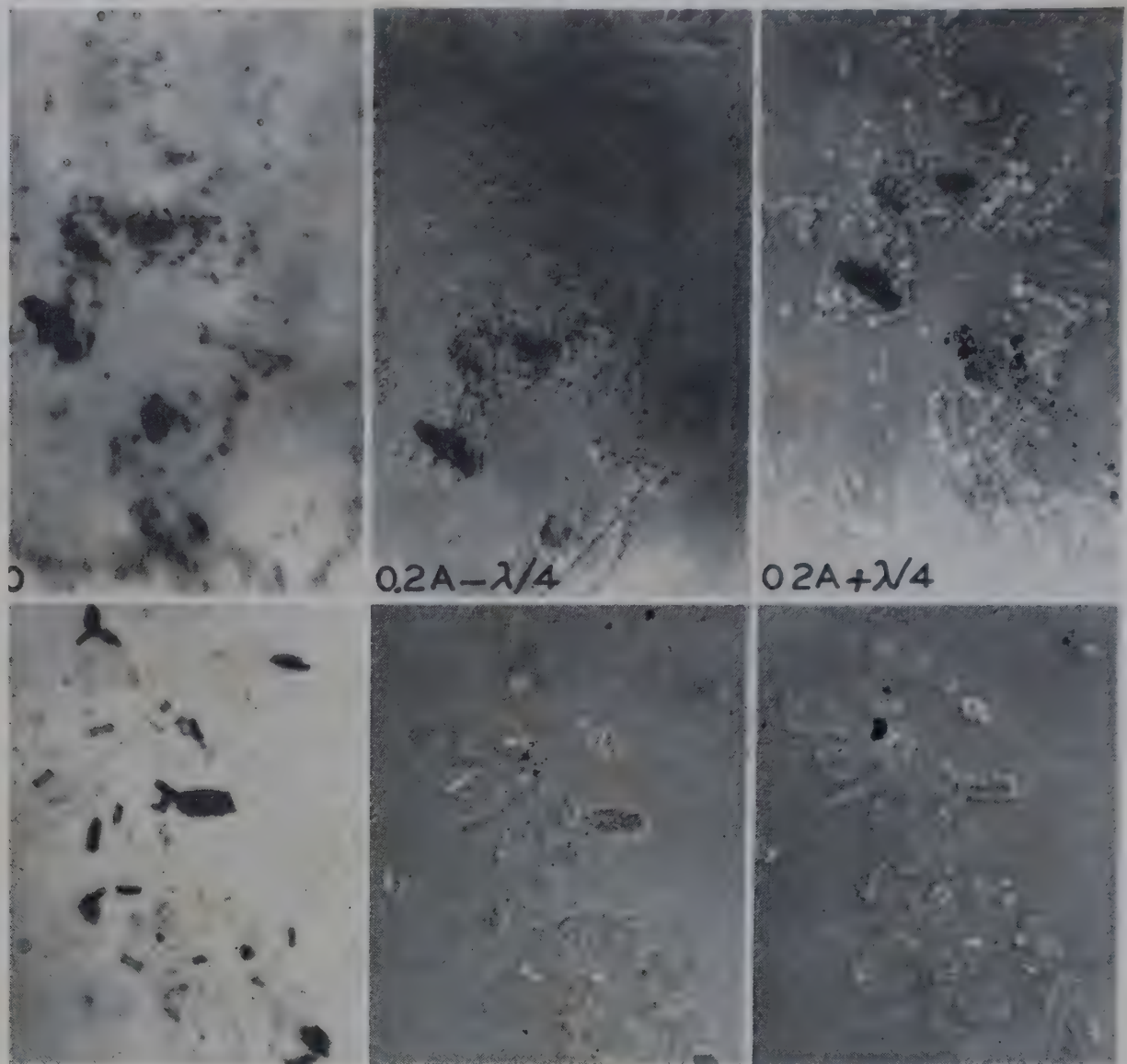


Fig. 9.—Photomicrographs of urinary sediments from a case of nephritis showing difference in detail between microscopic picture without phase and with phase (dark and bright contrast). (Courtesy American Optical Co., Scientific Instrument Division.)

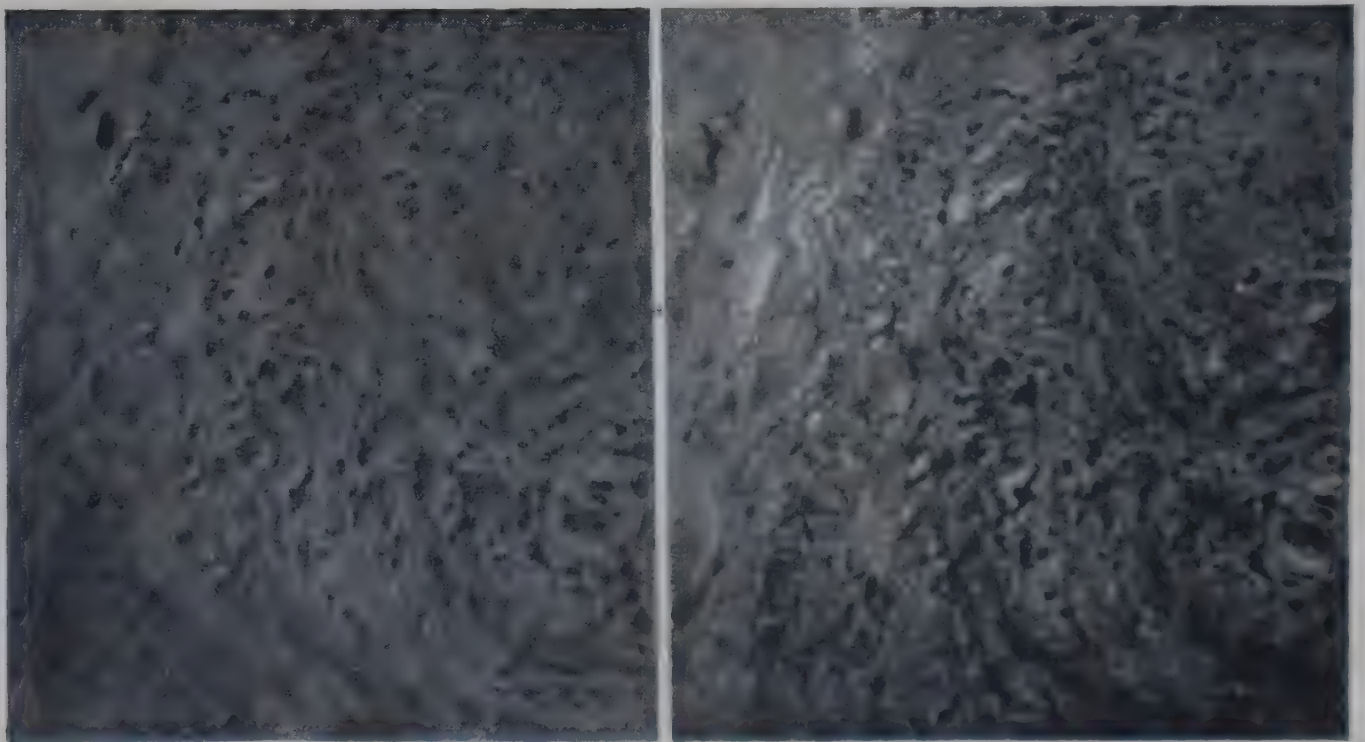


Fig. 10.—Photomicrographs of section of liver (degeneration of liver) with brightfield microscope (left) and phase microscope (right). Note increased contrast obtained with the phase microscope. (Courtesy American Optical Co., Scientific Instrument Division.)

isms, effects of drugs on organisms (ciliary action, cyclosis, digestion, etc.) and possibly the changes involved in malignancy, with the use of the phase microscope.

The microscope has an application in biology³ for the identification of free living and parasitic bacteria, protozoa, and other microorganisms, counts of their population concentrations, the histology, cytology, the invasion and dissolution of cells such as bacteriophage on bacteria, and the differences to be found between unstained, living protoplasm and fixed and stained specimens.

With the phase microscope the contrast in the image may be increased or decreased, or reversed and increased or decreased. This is accomplished by means of diffraction plates inserted into the objective of the microscope and a centerable annular diaphragm added to the microscope condenser. Four types of diffraction plates are available in varying combinations of absorption and retardation. Bright contrast (specimen brighter than its surroundings) is useful for counting and observing small particles, and dark contrast (specimen darker than surroundings) for measuring. Some observers prefer dark contrast because the image is similar to that of stained material. Fig. 9 shows the increased contrast obtained with bright and dark contrast with nearly invisible urinary casts. The phase microscope is also useful for bringing out detail in stained preparations, Fig. 10.

The equipment may be used on many current models of Spencer microscopes. In addition to the diffraction plates and the condenser diaphragm, an auxiliary telescope is recommended for centering the equipment. It is not difficult to use the equipment. The usual microscopic preparation methods are used.

Thin preparations are, as usual, better than thick ones. Little gain may be expected with strongly stained preparations with high contrast, but less contrasty specimens may be brought out more sharply, especially unstained living cells and tissues. The phase microscope offers the only method for measuring living microorganisms, because they can be photographed with speed light sources with sharp margins while unstained and in a suitable culture fluid. Staining and other methods, chemical action, dehydration, etc., alter their size.

Inquiries should be directed to the American Optical Company, Scientific Instrument Division, Buffalo 15, N. Y.

ANALYTICAL BALANCE

The analytical balance is an important part of the medical laboratory. There should be a good working balance in every laboratory, accurate down to at least 0.1 mg. It is necessary to know at all times whether the balance is in satisfactory weighing condition. Kreider⁴ gives the following points regarding adjustment of such a balance:

1. The general condition of the balance must be acceptable.
2. The rest point should be constant for any particular load when the mass on each side of the pans is equal.

³Cold Spring Harbor, Symposia on Quantitative Biology 11: 1947.

⁴Kreider, Leonard C.: Ind. & Eng. Chem. 18: 2, 1941. Craig, A.: Ind. Eng. Chem., Anal. Ed. 11: 581, 1939.

3. The balance must be of the proper degree of sensitivity. (Sensitivity is understood to be numerically equal to the deflection on the pointer scale caused by the addition of a 1-mg. load to a single pan of the balance.)
4. The balance must give weighings that are closely reproducible.
5. The balance must have lever arms of nearly equal length.

These points are intimately interrelated; for example, an imperfect knife-edge may cause the balance to perform poorly in respect to points 2, 3, and 4.

Point 1 is not readily tested qualitatively, but a careful inspection will usually suffice.

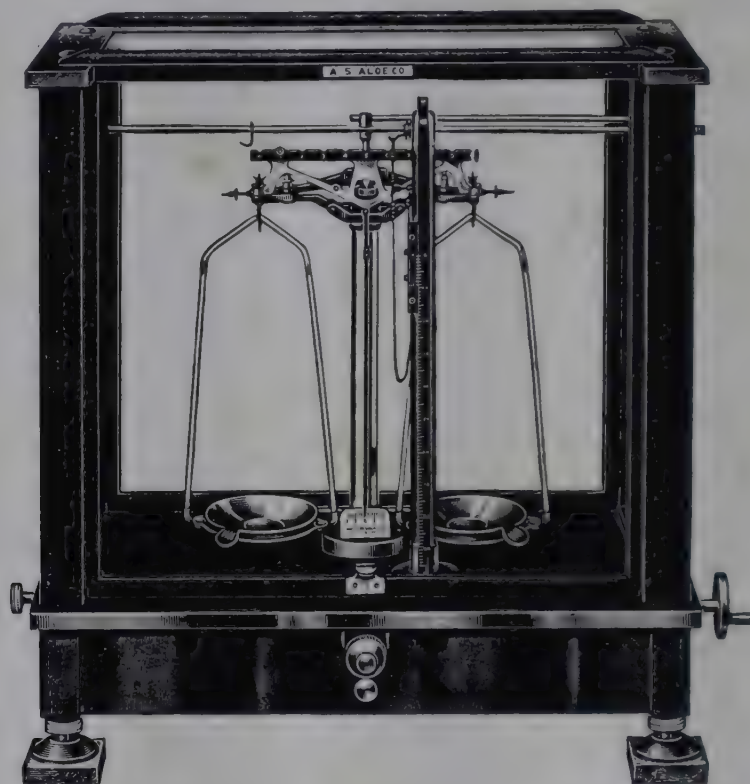


Fig. 11.—Ainsworth analytical chain balance. (Courtesy Aloe Scientific, St. Louis, Mo.)

Make sure that the beam releases, pan rests, rider carrier, chain weight devices, and other moving parts are in good mechanical condition. See that the knife-edges are separated from their bearing plates by the beam lift to a gap of about 0.1 mm.; and that when the beam lift is released all three knife-edges make contact with their plates over the whole edge gently and simultaneously. This is essential to the life of the edges. Metal parts well finished and free from corrosion are desirable, but do not necessarily indicate an accurate balance.

The information sought in points 2 and 3 can be obtained quantitatively by the following method:

Place the balance on a firm support in a part of a room where a fairly constant temperature prevails (away from radiators, open windows, and other drafts, out of direct sunlight, and removed from other hot light sources). Level the balance with the set-screws provided in the base. If the balance has recently been moved from another location, open the door of the case and allow at least an hour for the balance to attain room temperature.

After the balance has been brought to the same temperature as its environment, determine the data required to construct a table similar to Table 1. For this purpose select two sets of analytical weight, W_1 and W_2 . It is convenient,

but not necessary, to have the sets agree within fairly narrow limits. They need not be calibrated. If two sets of weights are not available, one can make shift with only one set; for example, if the data at 80 grams' load are to be determined, one could call the two 10-gram weights together W_1 and the 20-gram weight W_2 .

TABLE 1. DATA FOR STUDENT BALANCE

WEIGHT OF W_1 AND W_2 (EACH) GRAMS	REST POINT (a), A	REST POINT (b), B	AVERAGE REST POINT, C (A + B)/2	REST POINT (c), D	SENSITIVITY E (B - D)
0	9.0	9.0	9.0	6.9	2.1
10	9.5	9.1	9.3	7.3	1.8
20	10.2	8.6	9.4	7.2	1.4
50	9.7	9.5	9.6	8.5	1.0
100	9.3	11.1	10.2	10.3	0.8

- (a) W_1 on left pan, W_2 on right pan.
- (b) W_2 on left pan, W_1 on right pan.
- (c) W_2 on left pan, $W_1 + 1$ mg. on right pan.

Table 1 records data obtained in applying this method to a typical student balance. The principle involved is that of double weighings first devised by Gauss.

Constancy of the values in column C would satisfy point 2. If the rest point in C should shift by as much as two or three pointer scale divisions between loads of zero weight and 100 grams' weight on each pan, the balance would not be acceptable for determining absolute mass values, but might prove acceptable for certain types of gravimetric analysis where the determination of small differences in mass only is required. The balance tested (Table 1), where the rest point shifts 1.2 scale divisions between zero weight and 100 grams' weight load, on each pan on the basis of a sensitivity of 0.8 at 100 grams' load, would cause an error of 1.5 mg. in determining a 100-gram load. This amounts to a deviation of only 0.0015 per cent, which would be negligible for most work.

It is worth while to test the effect of changing the position of the masses from the centers of the pans to the edges and see if the value of the rest point is thereby changed. Defects of the end knife-edges may sometimes be detected by this method, whereas they may remain unnoticed when the masses on the pans are perfectly centered. The point of rest should also be checked by using swings of small amplitude and then swings of considerably greater amplitude. Difference between the two values indicates worn, nonparallel, or otherwise faulty knife-edges.

If the balance is to be used where the requirements are only moderately exacting (point 3), the sensitivity (column E) should have a numerical value of at least 2 and preferably 3 or 4 at zero load on the balance pans. The sensitivity of a balance should remain nearly constant or should decrease slowly and regularly with increasing load on the balance pans. The fall in sensitivity is usually due to a difference in level between the middle and the end knife-edges, and may be caused by bending of the beam under the load, by wear of the knife-edges, or by not sharpening them uniformly. In general, it is not safe to use a balance for loads that reduce the sensitivity to less than 40 per cent of the value with zero load. Weights of 90 to 100 grams should be the maximum allowed on each pan of the balance that is serving as our example.

Point 4 could be determined by again getting the rest point of the balance with pans empty after securing the data of Table 1. If this checks with the corresponding value in the table within 0.2 pointer scale division, the balance is satisfactory. If further checks on this point are desired, one can repeat the weighings at any pan load.

Point 5 is easily tested as follows:

An object of mass M (a 20-gram weight is convenient) is placed on the left-hand balance pan and counterbalanced with other weights from the set and their sum, S , is recorded.

M is then transferred to the right-hand balance pan and again counterbalanced with weights from the set and their sum, S' , is recorded. If L equals the length of the left lever arm and R equals the length of the right lever arm of the balance, from the principle of the lever

$$ML = RS \quad (1)$$

and

$$MR = LS' \quad (2)$$

If we divide Equation 2 by Equation 1 we get

$$R/L = \sqrt{S/S'} \quad (3)$$

On developing the quantity under the radical sign in terms of a series of powers of x and S , where x represents the difference between S' and S ($S' = S \pm x$), we get the series

$$\frac{R}{L} = 1 \pm \frac{x}{2S} \pm \frac{x^2}{8S^2} \pm \frac{x^3}{16S^3} \quad (4)$$

In applying this to the balance, x is always very small as compared to S ; so the equation reduces essentially to

$$\frac{R}{L} = 1 \pm \frac{x}{2S} \quad (5)$$

The upper sign is used where S' is greater than S and the lower sign is used when S' is less than S . In a good balance the R/L value should be 1.0 ± 0.00002 . In dealing with comparative values, as in gravimetric analysis, an R/L value of 1.0 ± 0.0002 can be tolerated without appreciable error in the final result.

In deciding whether a balance is suitable for the work at hand, one must also know the probable limits of error introduced by factors other than the balance. The balance may be used without hesitation if it is twice as accurate as the least accurate of any of the other measurements involved. It is probable that more errors in student and commercial work are due to uncalibrated or poorly calibrated weights than to inaccurate balances. Moreover, manipulative techniques, aside from weighing, usually introduce far larger errors than can be accounted for by the inaccuracy of weighing; and the percentage of error of many analytical methods, due to such things as end point errors, solubility of precipitate, adsorption, deliquescence, inability to measure volumes accurately, etc., is far greater than most of us would tolerate in an analytical balance.

How to Use an Analytical Balance.—For all accurate analytical work, a balance which is sensitive to a tenth of a milligram should be used. In order to maintain the sensitivity and accuracy of such an instrument there are certain rules which must be observed in regard to the weighing of materials or objects and the care of the balance. An analytical balance must be perfectly level before it can be used. If it is not level it may be made so by adjusting screws at the corners of the base. A plumb bob or a spirit level may be used to ascertain whether or not the balance is level.

The case should be kept closed at all times except when adding or removing weights and material. When the balance is not in use it should be kept covered in order to prevent fumes or dust from getting into the case. Care should be exercised to avoid spilling any of the reagents being weighed upon the pan supports or upon the floor. If any dry material is spilled, it should be removed with a camel's hair brush. If any deliquescent material is spilled, it should be removed with a damp cloth, and if any liquid is spilled, it should be removed with a soft cloth.

Before the balance is used it must be checked to see whether or not it is in balance. This may be ascertained by releasing the beam and pan supports and observing the pointer swings. If any change is to be made the thumb screws at the ends of the beam may be moved. If the right hand pan is heavier the thumb screw may be moved in toward the center, virtually shortening that arm. If the reverse is true the thumb screw may be moved out from the center so that the arm is lengthened.

Dry materials may be weighed in weighing bottles, upon watch glasses or weighing pan provided for that purpose. Liquids must be weighed in weighing flasks or bottles. The material being weighed should be at room temperature. If the temperature of the material is considerably above room temperature air currents will be created which will materially influence the accuracy of the weighing. If crucibles contain material which takes up moisture from the air they should be placed in a desiccator before being weighed. If the material gains considerable weight in the weighing process the crucible should be reheated and reweighed quickly with the approximate weights placed upon the pan before the material is removed from the desiccator. A cooling time should be allowed in order to bring the temperature of the material to the temperature of the room before reweighing.

The material to be weighed is always placed upon the left hand pan of the balance. The weights to be used are always placed upon the right hand pan. When extreme accuracy is necessary the material being weighed may be removed from the pan and weights or sand may be used to counterbalance the weights which have already been used and the average of the weights taken.

Before the addition or removal of any material or weights the beam and pans must be supported by the supports so provided. The pan rests and beam supports should be lowered carefully in order to avoid damage to the knife-edges. The weights should be handled with forceps only. Any moisture upon the fingers will materially influence the weighing of material if the weights are handled with the fingers. Natural oils or fats from the skin will also influence weighing if the weights are handled with the fingers.

All weights should be carefully counted; in order to avoid errors they should be counted twice, once when they are removed from the box and again when they are removed from the pan preparatory to replacing them in the box.

In weighing, the pointer need not come to absolute rest at the mid-point of the scale but the point of balance may be determined by observing the swings of the pointer to the right and left of the mid-point. If the pointer swings an equal number of spaces to the right and to the left of the mid-point the point of balance may be considered as having been reached.

The sensitivity of the balance may be determined at intervals according to the outline on pages 15 and 16.

METRIC WEIGHTS AND MEASURES

The metric system is based on a system of "tens"; that is, each succeeding unit is ten times the preceding one. This system of weights and measures depends on the *meter*, the standard unit of length. One thousand meters is called a *kilometer*; 100 meters, a *hectometer*; 10 meters, a *decameter*; one-tenth of a meter, a *decimeter*; one one-hundredth of a meter, a *centimeter*; and one one-thousandth of a meter, a *millimeter*.

The unit of weight is the *gram*, or the weight of 1 cubic centimeter of distilled water at 4° C. at sea level (760 mm.). The *kilogram* is 1000 grams; the *hectogram* is 100 grams; the *decagram* is 10 grams; the *decigram* is one-tenth of a gram; the *centigram* is one one-hundredth of a gram; the *milligram* is one one-thousandth of a gram.

The unit of capacity is the *liter*. The most commonly used metric unit of volume in the laboratory is the *milliliter* (ml.), which is one one-thousandth of a liter, or one cubic centimeter (c.c.). A cubic millimeter is one one-thousandth of a cubic centimeter.

Fractions of measurements are given Latin prefixes; multiples of measurements are given Greek prefixes.

1 meter				=	39.37 inches
1 decimeter	=	0.1 meter	=	10 centimeters	= 3.937 inches
1 centimeter	=	0.01 meter	=	10 millimeters	= 0.3937 (2/5) inch
1 millimeter	=	0.001 meter	=	0.1 centimeter	= 0.03937 (1/25) inch
1 micron (μ)	=	0.001 millimeter			= 1/25,000 inch

There are approximately 2.54 centimeters to an inch. To convert inches to centimeters, multiply the number of inches by 2.54. To convert centimeters to inches, divide the number of centimeters by 2.54.

There are approximately 25.4 millimeters to an inch. To convert inches to millimeters, multiply the number of inches by 25.4. To convert millimeters to inches, divide the number of millimeters by 25.4.

There are approximately 25,000 microns to an inch. To convert microns to inches, divide the number of microns by 25,000. To convert microns to millimeters, divide the number of microns by 1,000.

To convert millimeters to centimeters, divide by 10. To convert centimeters to millimeters, multiply by 10.

1 square centimeter = 100 square millimeters = 0.155 sq. in.
1 cubic centimeter = 1000 cubic millimeters

1 gram	= 15.43 grains	= 0.563 dram (avoir.)	= 1000 milligrams
		= 0.257 dram (apoth.)	
1 decigram	= 0.1 gram	= 10 centigrams	= 100 milligrams
1 centigram	= 0.01 gram	= 0.1 decigram	= 10 milligrams
1 milligram	= 0.001 gram	= 0.1 centigram	
1 gamma	= 0.001 milligram	= 1 microgram, designated by the symbol γ (gamma)	
1 kilogram	= 1000 grams		

1 liter = 1000 cubic centimeters (c.c.)

1 cubic centimeter	= 0.001 liter (milliliter)	= 1000 cubic millimeters
1 cubic millimeter	= 0.000001 liter	= 0.001 cubic centimeter

1 ounce	= 28.35 (appr. 30) grams (avoir.)
1 pound	= 454.60 (appr. 500) grams (avoir.)

To convert	grams to milligrams	multiply by 1000
To convert	milligrams to grams	divide by 1000
To convert	c.c. to c.mm.	multiply by 1000
To convert	c.mm. to c.c.	divide by 1000
To convert	decigrams to milligrams	multiply by 100
To convert	centigrams to milligrams	multiply by 10
To convert	minims to c.c.	multiply by 0.061
To convert	grains to grams	multiply by 0.0648
To convert	grams to grains	multiply by 15.43
To convert	c.c. to minims	multiply by 16.23
To convert	fluidounces to c.c.	multiply by 29.57
To convert	c.c. to fluidounces	divide by 29.57

To convert degrees Centigrade to degrees Fahrenheit, multiply degrees Centigrade by 1.8 (°) and add 32 to the result.

Example.—

60° C. $60 \times \frac{9}{5} = 108^\circ$ $108^\circ + 32^\circ = 140^\circ$ F.

To convert degrees Fahrenheit to degrees Centigrade, subtract 32 from degrees Fahrenheit and multiply the remainder by $\frac{5}{9}$.

Example.—

98.6° F. $98.6^\circ - 32^\circ = 66.6^\circ$ $66.6^\circ \times \frac{5}{9} = 37^\circ$ C.

TABLE 2. INTERNATIONAL ATOMIC WEIGHTS, 1952

		ATOMIC	ATOMIC			ATOMIC	ATOMIC
	SYMBOL	NUMBER	WEIGHT*		SYMBOL	NUMBER	WEIGHT*
Actinium	Ac	89	227	Neon	Ne	10	20.183
Aluminum	Al	13	26.98	Neptunium	Np	93	[237]
Americium	Am	95	[243]	Nickel	Ni	28	58.69
Antimony	Sb	51	121.76	Niobium	Nb	41	92.91
Argon	A	18	39.944	(Columbium)			
Arsenic	As	33	74.91	Nitrogen	N	7	14.008
Astatine	At	85	[210]	Osmium	Os	76	190.2
Barium	Ba	56	137.36	Oxygen	O	8	16
Berkelium	Bk	97	[245]	Palladium	Pd	46	106.7
Beryllium	Be	4	9.013	Phosphorus	P	15	30.975
Bismuth	Bi	83	209.00	Platinum	Pt	78	195.23
Boron	B	5	10.82	Plutonium	Pu	94	[242]
Bromine	Br	35	79.916	Polonium	Po	84	210
Cadmium	Cd	48	112.41	Potassium	K	19	39.100
Calcium	Ca	20	40.08	Praseodymium	Pr	59	140.92
Californium	Cf	98	[246]	Promethium	Pm	61	[145]
Carbon	C	6	12.010	Protactinium	Pa	91	231
Cerium	Ce	58	140.13	Radium	Ra	88	226.05
Cesium	Cs	55	132.91	Radon	Rn	86	222
Chlorine	Cl	17	35.457	Rhenium	Re	75	186.31
Chromium	Cr	24	52.01	Rhodium	Rh	45	102.91
Cobalt	Co	27	58.94	Rubidium	Rb	37	85.48
Copper	Cu	29	63.54	Ruthenium	Ru	44	101.7
Curium	Cm	96	[243]	Samarium	Sm	62	150.43
Dysprosium	Dy	66	162.46	Scandium	Sc	21	44.96
Erbium	Er	68	167.2	Selenium	Se	34	78.96
Europium	Eu	63	152.0	Silicon	Si	14	28.09
Fluorine	F	9	19.00	Silver	Ag	47	107.880
Francium	Fr	87	[223]	Sodium	Na	11	22.997
Gadolinium	Gd	64	156.9	Strontium	Sr	38	87.63
Gallium	Ga	31	69.72	Sulphur	S	16	32.066
Germanium	Ge	32	72.60				±0.003
Gold	Au	79	197.2	Tantalum	Ta	73	180.88
Hafnium	Hf	72	178.6	Technetium	Tc	43	[99]
Helium	He	2	4.003	Tellurium	Te	52	127.61
Holmium	Ho	67	164.94	Terbium	Tb	65	159.2
Hydrogen	H	1	1.0080	Thallium	Tl	81	204.39
Indium	In	49	114.76	Thorium	Th	90	232.12
Iodine	I	53	126.91	Thulium	Tm	69	169.4
Iridium	Ir	77	193.1	Tin	Sn	50	118.70
Iron	Fe	26	55.85	Titanium	Ti	22	47.90
Krypton	Kr	36	83.80	Tungsten	W	74	183.92
Lanthanum	La	57	138.92	(Wolfram)			
Lead	Pb	82	207.21	Uranium	U	92	238.07
Lithium	Li	3	6.940	Vanadium	V	23	50.95
Lutetium	Lu	71	174.99	Xenon	Xe	54	131.3
Magnesium	Mg	12	24.32	Ytterbium	Yb	70	173.04
Manganese	Mn	25	54.93	Yttrium	Y	39	88.92
Mercury	Hg	80	200.61	Zinc	Zn	30	65.38
Molybdenum	Mo	42	95.95	Zirconium	Zr	40	91.22
Neodymium	Nd	60	144.27				

*A value given in brackets denotes the mass number of the most stable known isotope. Names enclosed in parentheses may also be used.

GENERAL FACTS ON SOLUTIONS

For clinical laboratory procedures solutions of various kinds are employed. These solutions must be carefully made in order that dependable results may be obtained with them. If solutions are to be accurate, the dry reagents must be weighed upon an accurate analytical balance or if the solution is not to be a volumetric one, the dry ingredients may be weighed upon an accurate trip balance, unless the quantity is very small. Liquids must be measured accurately, either by using volumetric pipettes or volumetric flasks. Dry reagents



Fig. 12.—The Aristocrat refrigerated model centrifuge, model L-704, with built-in tachometer and automatic timing device. (Courtesy Phillips-Drucker, St. Louis, Mo.)

should be transferred to a beaker from the weighing pan or watch glass and a small amount of the solvent added in order to avoid the possible loss of any of the material. The watch glass should then be rinsed with a small amount of the solvent and the rinsings added to the beaker in order to carry into the receptacle all of the solute. After dissolving the dry ingredients with a small amount of the solvent, it is transferred quantitatively to a volumetric flask for final dilution. The beaker is rinsed several times with the solvent and the combined rinsings are placed in the volumetric flask.

Of the several solutions used for laboratory work, the per cent solution is perhaps the most commonly employed. Such solutions may be made by weighing (in grams) the quantity of dry ingredients indicated by the figure which precedes the per cent sign, and adding enough diluent to bring the volume to the expressed total (100 c.c. being one complete quantity).

Molar Solutions (written M) contain one mole or one gram molecular weight of a solute diluted to 1 liter of solution. If water of crystallization is included in the formula, it, too, must be added to the total molecular weight.

Example.—Sodium chloride has the formula NaCl.

Na	-----	22.997	
Cl	-----	35.457	
NaCl	-----	58.454	gram weight or mole. Therefore,

a molar solution of sodium chloride is one which contains 58.454 grams of sodium chloride in a liter of solution.

M/10 expresses a tenth molar solution, or $\frac{1}{10}$ the strength of a molar solution. 2M expresses twice the strength of a molar solution, or 2 moles.

Normal Solutions (written N).—Normal solutions are considered the basis of all laboratory tests and solutions. In volumetric analysis the technician or chemist must have some solutions of known strength to use in determining the concentrations of many different substances.

In order to make this accurate volumetric chemical work more efficient, chemists have devised the system of normal solutions.

A **normal solution** is a solution which contains one gram of ionizable or replaceable hydrogen or its equivalent in 1 liter of solution.

By equivalent weight is meant the weight in grams of any substance which can replace, unite with, or have the same reacting value as one atomic weight of hydrogen (1.008 gm.).

Therefore, one liter of one kind of normal solution will have the same reacting value as one liter of any other kind of normal solution; in other words, normal solutions react c.c. for c.c. with each other.

To figure the amount of chemical to be dissolved in a total of 1 liter of solution, first figure the molecular weight. If water of crystallization is included in the formula on the label of the bottle, that must be added to the formula for the molecule when determining molecular weight. Divide this weight by the valence of the radical to obtain grams per liter. Weigh all chemicals accurately on an analytical balance and use volumetric flasks to make the final dilutions. Crystalline cupric sulphate has the formula $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

The molecular weight is

Cu	-----	63.54	
S	-----	32.06	
O ₄	-----	$16 \times 4 =$	64.00
			<hr/> 159.60
5 H ₂ O =			
	H ₂ = $2 \times 1.008 =$	2.016	
	O =	16.	
		<hr/> 18.016	$\times 5 = 90.080$
		159.60	
		<hr/> 90.08	
		249.68,	molecular weight.

The radical SO_4 has a valence of 2. Divide the molecular weight of cupric sulphate by 2 to determine the number of grams to be dissolved in a liter of solution. $249.68 \div 2 = 124.84$ gm.

Use 124.84 gm. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per liter of solution to make a N/1 solution of cupric sulphate; or use 249.68 (the molecular weight) to make a M solution.

To make a normal solution of a liquid, divide the molecular weight of the liquid to be used by the valence of the radical; divide this result by the specific gravity of the substance; then divide this result by the concentration of the liquid. Or use the following formula:

$$\text{c.c. per liter} = \frac{\text{Molecular weight}}{\text{Valence} \times \text{Specific Gravity} \times \text{Concentration}}$$

To Make a Normal Solution of Hydrochloric Acid

Hydrochloric acid has the formula HCl. First determine the molecular weight of HCl.

H	-----	1.008
Cl	-----	35.457
		<hr/> 36.465

The specific gravity and concentration of hydrochloric acid can be found on the label on the bottle. Assume that the specific gravity is 1.19 and that the concentration is 38%. The valence of HCl is 1. Using the formula given above:

$$\frac{36.465}{1 \times 1.19 \times 0.38} = 80.6.$$

Place 500 c.c. of distilled water in a liter volumetric flask.

Add 80.6 c.c. of concentrated hydrochloric acid and mix.

Dilute to exactly 1000 c.c. with distilled water and mix thoroughly. This solution must now be titrated against a normal alkali and standardized in the manner described below.

To Make a Normal Solution of Sulphuric Acid

Sulphuric acid has the formula H_2SO_4 . The molecular weight of sulphuric acid must be divided by 2 when figuring the quantity of sulphuric acid needed to make a normal solution, because this substance has a valence of 2.

H ₂	-----	2 × 1.008 =	2.016
S	-----		32.06
O ₄	-----	4 × 16.00 =	64.00
H ₂ SO ₄	-----		<hr/> 98.076, molecular weight of H ₂ SO ₄ .

Specific gravity 1.83; concentration 92%; valence 2.

$$\frac{98.076}{2 \times 1.83 \times 0.92} = 29.1 \text{ c.c.,}$$

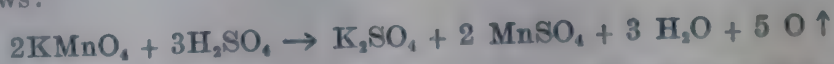
the amount of sulphuric acid to be used in making 1 liter of N/1 acid. (Specific gravities and concentrations of acids are written on the label when the chemicals come from the factory.) This solution must be titrated and standardized against a normal base before use, in the manner described below.

It is customary to make any normal solution of a liquid slightly stronger than the exact quantity figured and dilute according to titration results. See example below.

If an oxidizing agent is to be made, take into consideration the amount of oxygen to be liberated. Figure how many molecules liberate oxygen for X hydrogens, then divide the number of hydrogens by the number of molecules to obtain the number by which to divide the molecular weight, in other words, the hydrogen equivalent.

Preparation of Normal Solution of an Oxidizing Agent

Potassium permanganate will be used as an example of an oxidizing agent. Potassium permanganate in the presence of sulphuric acid and some easily oxidizable substance is decomposed as follows:



From the above equation it will be seen that 2 permanganate molecules liberate oxygen enough for 10 hydrogen atoms. If 2 molecules liberate enough oxygen for 10 hydrogen atoms, then 1 molecule must liberate enough for 5 hydrogen atoms. To make a normal solution of KMnO_4 , use $\frac{1}{5}$ of the molecular weight in enough distilled water to make one liter.

Titrate against a normal oxalic acid, with approximately 5 c.c. of normal sulphuric acid added. Keep the solution warm in the casserole (75° C.). The endpoint is a faint pink which remains.

To make a fraction of normality, divide the amount of substance to be used for a normal solution by the figure which represents part of normality; for example, N/10 means one-tenth of normality, and consequently only one-tenth of the amount for a normal solution will be used in this case. This is also written 0.1 N, tenth-normal, 1/10 N.

To make stronger-than-normal solutions, multiply the quantity determined for normality by the number indicating the strength desired. For example, 2 N means twice normal, and twice as much chemical is used.

To make weaker normalities from N/1 solutions, dilute in the usual manner, using all volumetric glassware. N/10 means 1 to 10 dilution, 2/3 N means 2 parts diluted to a total of 3, and 0.12 N means 12 parts diluted to 100 parts total.

Weigh the chemicals very accurately, and use all volumetric glassware when preparing normal solutions.

To Make a Normal Solution of a Base

Divide the molecular weight by the valence of the radical to obtain the number of grams of the substance per liter. Weigh this quantity accurately, transfer to a liter volumetric flask, dissolve, and dilute to 1000 c.c. with distilled water. Titrate against a normal acid and standardize as described below.

To Make a Normal Solution of a Salt

Determine the molecular weight of the salt, and divide by the valence of the radical. If water of crystallization is present, this must be added to the molecular weight before dividing by the valence.

To Make a Normal Solution of Sodium Hydroxide

To make a normal solution of sodium hydroxide after the molecular weight has been obtained as above outlined, a quantity slightly more than the molecular weight may be weighed for a concentrated solution and may be made in the following way. Sodium hydroxide has a quantity of Na_2CO_3 present and this may be removed for more accurate results. To remove this Na_2CO_3 , about 100 grams of sodium hydroxide sticks or pellets are weighed and dissolved in 100 c.c. of distilled water. The flask should be cooled under cold running water while the solution is being made. After the NaOH has dissolved, the flask is covered and allowed to stand for two or three days during which time the Na_2CO_3 settles out. The supernatant fluid then may be decanted or removed with a syphon. This clear solution is used to make the normal solution. To make a "too strong" solution, about 65 or 70 c.c. of the clear supernatant solution are diluted to 1000 c.c. with distilled water and this is used for the titration against a normal acid solution. To standardize this "too strong" alkali, two burets are set up. In one buret place the "too strong" alkali and in the other, the already titrated acid solution. Twenty c.c. of the "too strong" alkali may be measured into each of three beakers. Add 2 drops of indicator and titrate with the normal acid from the other buret. To calculate the correct dilution, use the following calculation:

$$\frac{20. \text{ (c.c. of alkali used)}}{\text{No. of c.c. of N/acid used}} \times 1000 = \text{c.c. of "too strong" NaOH to be diluted to 1 liter.}$$

Measure this amount and retitrate. Twenty c.c. should be neutralized by 20 c.c. of normal acid.

Example.—

First sample of alkali required	21.8 c.c. N acid
Second sample required	22.0 c.c. N acid.
Third sample required	21.9 c.c. N acid

The correct dilution is then calculated as follows:

$$\frac{20.0}{21.9} \times 1000 = 913.2 \text{ c.c. of the "too strong" alkali should be diluted to 1000 c.c.}$$

Standardization of Normal Solutions

Standardization of Sulphuric Acid, N/1

Principle.—

A stronger than normal solution of sulphuric acid is made and titrated against a N/1 base. It is then diluted according to calculation and titrated again to confirm the normality.

Methyl Orange Indicator.—

Dissolve 0.1 gm. of methyl orange in enough distilled water to make 100 c.c. solution. This indicator is stable for about one year.

Base.—

Dry a quantity of sodium carbonate, c.p., in the oven at 105° C. for 3 to 4 hours.

Weigh exactly 5.3 gm. and transfer to a 100 c.c. volumetric flask. Repeat this procedure three times. Each time, wash into the flask with distilled water, dissolve, and dilute to exactly 100 c.c. with distilled water.

Sulphuric Acid.—

Place in a 1000 c.c. Pyrex volumetric flask 500 c.c. distilled water.

Add 30 c.c. concentrated sulphuric acid, c.p., slowly and with constant mixing. Cool under running water while adding the acid. Cool and dilute to 1000 c.c. with distilled water. Mix well.

Titration.—

Fill a 25 c.c. burette with the sulphuric acid solution.

Pipette in a casserole 20 c.c. of the normal sodium carbonate solution.

Add 1 drop of methyl orange indicator.

Add the sulphuric acid cautiously from the burette, stirring constantly until the first orange color which remains on stirring. This is the endpoint.

Read from the burette the c.c. of sulphuric acid used.

Repeat the titration with each batch of sodium carbonate solution. The three titrations should check within 0.1 c.c. of each other.

If more than 20 c.c. of sulphuric acid were required to neutralize the sodium carbonate solution, the sulphuric acid is too weak and more of the concentrated sulphuric acid should be added to the stock until the solution is stronger than normal. Then titrate again.

If less than 20 c.c. were required, it is too strong. Use the following formula to figure how much water to add to the sulphuric acid before mixing and repeating the titration.

Let T = the titration in c.c.

V = the total volume of acid solution remaining after the titration.

W = the c.c. of distilled water to be added to the solution.

$$\frac{V}{T} \times (20 - T) = W$$

Example.—

18.1 c.c. sulphuric acid used in the titration.

928 c.c. solution remains after the titration.

$$\frac{928}{18.1} \times (20 - 18.1) = 97.4 \text{ c.c. of distilled water to be added.}$$

Titration of Normal Base

Carry out the titration in the same manner as for sulphuric acid, using a standardized acid in the casserole, and placing the alkali in the burette.

Normality Factors

Normality factors may be computed by determining the ratio between the number of c.c. of solution that has been used and the number of c.c. that should have been used.

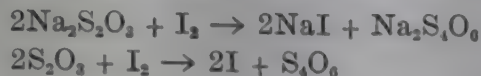
Normal Solutions in Ampules

There are now available on the market sealed glass or, when required, paraffin ampules containing definite quantities of reagents from which accurate normal solutions may be made. These reagents and solutions are stable indefinitely. To prepare a normal solution from one of these ampules, transfer the contents to a volumetric flask, wash quantitatively into the flask, and dilute to volume. These ampules may be obtained from the Will Corporation, 39

Russel St., Rochester 3, N. Y., or 596 Broadway, New York, N. Y. They are marketed under the name "Acculute." The author has tested some of these reagents against standard reagents made in his own laboratory and has found them very accurate.

Standardization of Solutions Used in Iodimetry

The fundamental reaction in iodimetric methods is the conversion of sodium thiosulphate and free iodine to sodium tetrathionate and sodium iodide, according to the following formula:



With this method free iodine and oxidizing agents which liberate iodine from iodides can be determined. Starch is used as an indicator. It turns blue with free iodine; the color vanishes when all free iodine has been combined.

Starch Indicator.—

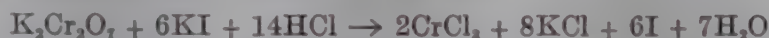
To 50 c.c. boiling distilled water

add 1 gm. of soluble starch and stir with a glass stirring rod until solution is complete.

Dilute to 100 c.c. with cold distilled water. To prevent the development of molds, dissolve 1 gm. of salicylic acid in the water before using it in the starch solution.

N/10 Potassium Dichromate Solution.—

Molecular weight of potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) is 294.20. The following equation shows how iodine is liberated from iodine by this salt.



It can be seen from the equation that one molecule of potassium dichromate liberates 6 atoms of I from KI; each atom of I is equivalent to 1 atom of hydrogen; thus a normal solution of potassium dichromate would contain one-sixth its molecular weight, in grams, per liter. A N/10 solution would, therefore, contain 4.903 grams of potassium dichromate per liter. Make the solution with the usual precautions, after having dried the dichromate in a desiccator. This solution is stable for several months if kept in a tightly closed bottle.

N/10 Sodium Thiosulphate.—

The molecular weight of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ is 248.20.

Make a solution slightly stronger than N/10 by dissolving 26 grams of sodium thiosulphate (of the above formula) in distilled water in a liter volumetric flask, and dilute to 1000 c.c. with distilled water. Titrate against N/10 potassium dichromate using potassium iodide as follows:

Titration of N/10 Sodium Thiosulphate.—

Place in a 600 c.c. beaker

50 c.c. distilled water

10 c.c. concentrated hydrochloric acid

3 gm. potassium iodide (KI) (weighed to the nearest 0.1 gm.).

Add 25 c.c. of N/10 potassium dichromate using a pipette. A brown color is produced by the liberation of I.

Cover the beaker, and allow it to stand in the dark for 5 minutes for the reaction to proceed.

Add 300 c.c. of distilled water and titrate with the N/10 sodium thiosulphate from a burette.

As soon as the brown color has faded, add 3 drops of starch indicator.

Titrate until the blue iodo-starch color disappears.

Read from the burette the c.c. of sodium thiosulphate used.

Adjust to normality in the usual manner by dilution, and confirm the results by re-titration. (See page 26.)

To prevent decomposition, if the solution is to be kept, add 10 c.c. of N/10 sodium hydroxide to the dissolved sodium thiosulphate before diluting it to 1 liter with distilled water. Such solutions are stable for about six months.

N/10 Iodine Solution.—

Balance a glass container on the trip balance, and weigh 13 gm. of solid iodine, re-sublimed.

Dissolve 30 gm. KI in 250 c.c. distilled water.

In a liter volumetric flask, mix the iodine and potassium iodide solution until the iodine is completely dissolved.

Dilute to 1000 c.c. with distilled water.

Titrate against 25 c.c. of N/10 sodium thiosulphate solution, adding 3 drops of starch indicator toward the end of the titration. Adjust in the usual manner.

A normal solution of I_2 contains 126.92 grams per liter.

Per Cent Solutions.—

Use volumetric glassware.

(1) If a per cent solution of a solid is desired.—

Dissolve the number of grams of the substance (indicated by the per cent desired) in enough solvent (water, alcohol, etc.) to make a total of 100 c.c. of solution.

Example.—To make a 10% solution of ferric chloride, dissolve 10 grams of ferric chloride in enough distilled water to make 100 c.c. of solution.

(2) If a per cent by volume of a liquid is desired.—

Add the correct number of c.c. of the liquid (indicated by the per cent desired) to solvent and dilute to 100 c.c. with the solvent.

Example.—To make a 5% solution of hydrochloric acid, add 5 c.c. of concentrated hydrochloric acid to distilled water and dilute to 100 c.c. with distilled water.

(3) If a per cent by weight of a liquid is desired.—

Dilute in the proportion of the per cent desired to the concentration of the liquid.

Example.—To make a 5% solution by weight of hydrochloric acid, dilute 5 c.c. of concentrated hydrochloric acid to a total of 36 c.c., since hydrochloric acid usually has a concentration of 36%. Add the acid to a little water and dilute to the total volume.

(4) To make any quantity of per cent solutions from other per cent solutions of stronger concentration, use the following equation:

$$S_1 : S_2 :: V_1 : V_2, \text{ where}$$

S_1 represents the strength of the weaker solution,

S_2 represents the strength of the stronger solution,

V_1 represents the volume of the stronger solution to be used, and

V_2 represents the total volume to which the solution is to be diluted.

Example.—To make 500 c.c. of 5% hydrochloric acid by weight (concentration 36%).

$$\begin{aligned} 5 : 36 &:: V_1 : 500 \\ 36 V_1 &= 2500 \\ V_1 &= 69.44 \end{aligned}$$

Dilute 69.44 c.c. of concentrated hydrochloric acid to 500 c.c. with distilled water, carrying out the usual precautions for adding an acid to water.

(5) To determine how much solution can be made from a definite amount of a per cent solution, use the same formula.

Example.—To determine how much 1% glucose solution can be made from 5 c.c. of 10% glucose,

$$\begin{aligned} 1 : 10 &:: 5 : V_2 \\ V_2 &= 50 \end{aligned}$$

Dilute the 5 c.c. of 10% glucose solution to 50 c.c. with distilled water.

MILLIEQUIVALENTS PER LITER

Gamble* introduced the conversion of the blood electrolytes into milliequivalents per liter. The formula for this conversion is:

$$\frac{\text{Concentration in mg. per 100 c.c. serum or plasma} \times 10 \times \text{valence}}{\text{Atomic weight}} = \text{meq./L. (milliequivalents per liter).}$$

*Chemical Anatomy, Physiology, and Pathology of Extracellular Fluid, Cambridge, 1947. Harvard University Press.

For example, let us suppose that 11 mg. calcium per 100 c.c. serum were found. This value expressed in milliequivalents will be as follows, the valence of calcium being 2, and its atomic weight being 40: $\frac{11 \times 10 \times 2}{40} = 5.5 \text{ meq./L.}$

One meq./L. of CO₂ occupies, under standard conditions, 22.26 c.c. Thus 1 meq./100 c.c. of CO₂ occupies 2.22 c.c. The volume of CO₂ in meq./L. is obtained, therefore, by dividing the CO₂ concentration in c.c. per 100 c.c. fluid by 2.22.

Gamble, using values expressed in meq./L., illustrated the acid-base equilibrium of blood plasma as:

BASES		ACIDS	
Na'	142	HCO ₃ '	27
K'	5	Cl'	103
Ca''	5	HPO ₄ ''	2
Mg''	3	SO ₄ ''	1
		Organic acids	6
		Proteins	16
	155		155

For further details, consult the chapter on Blood Chemistry.

EXPLANATION OF HYDROGEN ION CONCENTRATION

Determination of hydrogen ion concentration has a number of applications in laboratory procedures.

pH Scale

According to the Fahrenheit thermometer, 32° represents the freezing point of water and values above and below 32° represent degrees of heat and coldness, respectively. Above 32° means an increase in heat and below 32° means an increase in coldness.

We read the degree of acidity or alkalinity of a solution on the hydrogen ion scale in the same way. Instead of calling the points of difference degrees, in the hydrogen ion method we call these units pH values. A value of pH 7.0 means neutrality. Any pH higher than pH 7.0, such as 7.2, 7.4, 8.0, etc., means alkalinity. Figures below pH 7.0, such as 6.8, 6.6, 6.0, etc., mean acidity. Thus, a solution with a pH 7.6 is alkaline. A solution with a pH of 6.8 is acid and one of 6.4 is more acid.

Indicators

The same indicator must be used in standardizing the solution as in subsequent analyses. For the titration of strong mineral acids an indicator like alizarin or methyl red should be used. These change at about pH 6. Phenolphthalein is used only in titration of weak acids and changes at about pH 8.2. This indicator cannot be used in the presence of carbonic acid or ammonium salts. A mixed indicator is often useful as greater contrast in color is obtained, separated by a gray shade in the transition interval. Methyl red and methylene blue mixture changes from violet to green instead of red to yellow.

The measurement of pH is based upon the fact that various indicators change in color when acted upon by solutions of different acidities or alkalini-

ties. Litmus has been commonly used in the past for determining alkalinity or acidity of solutions, but it is very insensitive because it covers a wide range. For practical purposes, we must use indicators that cover very concisely the entire pH range from 1.2 to 9.6. Table 3 gives the principal indicators and their ranges and color changes.

TABLE 3

NAME	pH RANGE	COLOR CHANGE
Meta Cresol Purple	1.2—2.8	Red —Yellow
Bromphenol Blue	3.0—4.6	Yellow—Blue
Bromcresol Green	4.0—5.6	Yellow—Blue
Bromcresol Purple	5.4—7.0	Yellow—Purple
Chlorphenol Red	5.2—6.8	Yellow—Red
Bromthymol Blue	6.0—7.6	Yellow—Blue
Phenol Red	6.8—8.4	Yellow—Red
Cresol Red	7.2—8.8	Yellow—Red
Thymol Blue	8.0—9.6	Yellow—Blue
Methyl Red	4.2—6.3	Red —Yellow
Methyl Orange	3.1—4.4	Red —Yellow

Indicator papers are sometimes used to determine hydrogen ion concentration. For details see pages 43 and 44.

Rough Tests

In beginning the determination of the pH value of a given solution, when no information as to its pH value is available, make what is termed a “rough test.” To carry out this test, fill three or four graduated test tubes to the mark 10 c.c. with the solution to be tested. To the first one add 0.5 c.c. of bromthymol blue indicator solution, because it has a range of 6.0-7.6 and, therefore, covers the neutral point 7.0. This tells whether the solution is neutral, acid, or alkaline. Color change of bromthymol blue is from yellow at 6.0 to a deep blue at 7.6. If an intermediate color between yellow and deep blue is obtained, the pH value of the solution lies between 6.0 and 7.6, and hence it is either neutral or slightly acid or alkaline, in which case no further rough tests are necessary.

If a yellow color is obtained on adding the bromthymol blue indicator solution, the pH of the solution is at least 6.0 and may be lower. Even if the solution had a pH of 5.0 or 4.0, it would still give a yellow color with bromthymol blue. If, under these circumstances, a yellow color is obtained, 0.5 c.c. of the bromcresol green indicator solution is used, covering a more acid part of the range (that is from 4.0 to 5.6), which is added to the second test tube containing the solution of unknown pH. Color change with this indicator is from yellow at pH 4.0 to a deep blue at pH 5.6. If a color intermediate between yellow and deep blue is obtained with this test, the pH of the solution lies between 4.0 and 5.6. Thus, if bromcresol green (pH 4.0-5.6) gives a deep blue color and bromthymol blue (pH 6.0-7.6) a yellow color, it is apparent that the pH of the solution lies around pH 5.6-6.0; that is, within the range of chlorphenol red (pH 5.2-6.8). One indicator must be used in each tube: if bromthymol blue gives a yellow color, the bromcresol green must be added to a fresh tube. If a yellow color is obtained with bromcresol green, the test

should be repeated using bromphenol blue, covering a still more acid range, pH 3.0-4.6, etc. If, on adding bromthymol blue indicator in the first test, a deep blue color is obtained, the solution is alkaline and has a pH value of at least 7.6.. Repeat the test using thymol blue, which covers a more alkaline part of the range from pH 8.0-9.6.

The Accurate Test

If in using the bromthymol blue indicator, a color intermediate between yellow and deep blue is obtained, this shows that the pH of the solution lies between 6.0 and 7.6. Now prepare a set of bromthymol blue color standards. A set of bromthymol blue color standards consists of nine tubes and one tube of distilled water.

The color standards for bromthymol blue are simply nine tubes, containing 10 c.c. of solutions of definite pH value, that is, 6.0; 6.2; 6.4; 6.6; 6.8; 7.0; 7.2; 7.4; and 7.6; to each of which 0.5 c.c. of bromthymol blue indicator solution has been added. It is seen that these standards are made up in exactly the same manner in which the test was made, except that solutions of definite pH values were used. These color standards can be purchased, and it is not, therefore, necessary for the worker to make them up for himself.

The nine tubes are labeled with the pH values of the various solutions which they contain. The tube marked pH 6.0 will have a yellow color and that marked pH 7.6 a deep blue color. The intermediate tubes, which are marked 6.2, 6.4, 6.6, etc., will have colors intermediate between yellow and blue.

All that is now necessary is to match the test sample with the standards. When a match is obtained, the pH value of the solution is read off directly from the standard with which it matches.

If the rough test has shown the pH of the solution to lie between 5.2 and 6.8, a tube of the unknown, to which chlorphenol red indicator solution has been added, must, of course, be compared with a set of chlorphenol red color standards. These are similar to the bromthymol blue standards. Thus the tube marked pH 5.2 has a yellow color and that marked pH 6.8 has a deep red color. The intermediate tubes have colors intermediate between yellow and red. Analogously, if the pH of the solution were found to be between 4.0 and 5.6, the tube to which the bromcresol green solution was added must be compared with the bromcresol green color standards, etc.

It will be seen above that the ranges of the various indicators overlap; that is, bromthymol blue covers the range pH 6.0 to 7.6 and chlorphenol red the range pH 5.2 to 6.8. The values 6.0 to 6.8 are thus common to both indicators. It is, therefore, clear that if the pH value of the solution which is being tested lies between 6.0 and 6.8, determinations can be made with both indicators; that is, the sample to which the bromthymol blue indicator solution is added is compared with the bromthymol blue color standards, and the one to which the chlorphenol red is added is compared with the chlorphenol red color standards. As almost all of the indicators overlap, it is usually possible to make determinations with two indicators and thus check results.

Various color changes at various hydrogen ion concentrations together with the method of preparing the indicated solutions are given briefly below:

Thymol Blue (0.04%)

Acid Range

Red at 1.2-----Orange at 1.8-2.0-----Yellow at 2.8

Alkaline Range

Yellow at 8.2-----Gray at 8.8-9.0-----Blue at 9.8

Dissolve 100 mg. Thymol Blue

in 4.3 c.c. N/20 NaOH

Dilute to 250 c.c. with distilled water.

Bromphenol Blue (0.04%)

Yellow at 3.0-----Gray at 3.8-----Lavender at 4.6

Dissolve 100 mg. Bromphenol Blue

in 3 c.c. N/20 NaOH

Dilute to 250 c.c. with distilled water.

Methyl Red (0.02%)

Red at 4.4-----Orange at 5.2-----Yellow at 6.0

Dissolve 50 mg. Methyl Red

in 7.4 c.c. N/20 NaOH

Dilute to 250 c.c. distilled water.

Methyl red produces colors that are unstable. Unless read almost immediately after the addition of the indicator, the color changes so rapidly as to be of no value. For this reason, bromcresol green is frequently substituted for methyl red. The formula follows:

Bromcresol Green (0.04%)

Dissolve 100 mg. Bromcresol Green

in 2.86 c.c. N/20 NaOH

Dilute to 250 c.c. with distilled water.

Bromcresol Purple (0.04%)

Yellow at 5.4-----Gray at 6.0-6.2-----Purple at 7.0

Dissolve 100 mg. Bromcresol Purple

in 3.7 c.c. N/20 NaOH

Dilute to 250 c.c. with distilled water.

Bromthymol Blue (0.04%)

Yellow at 6.0-----Green at 6.6-6.8-----Blue at 7.6

Dissolve 100 mg. Bromthymol Blue

in 3.2 c.c. N/20 NaOH

Dilute to 250 c.c. with distilled water.

Phenol Red (0.02%)

Yellow at 6.6-----Orange at 7.2-----Red at 8.2

Dissolve 50 mg. of Phenol Red

in 2.85 c.c. N/20 NaOH

Dilute to 250 c.c. with distilled water.

Cresol Red (0.02%)

Yellow at 7.2-----Orange at 7.8-----Red at 8.8

Dissolve 50 mg. of Cresol Red

in 2.65 c.c. of N/20 NaOH

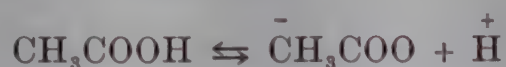
Dilute to 250 c.c. with distilled water.

Meaning of pH Values

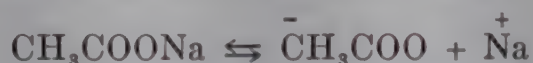
All liquids of which water is a constituent contain free H and OH ions. If the number of H ions exactly equals the number of OH ions, the solution is neutral. If the number of H ions exceeds that of the OH ions, the solution is acid; if it contains an excess of OH ions, it is alkaline. Acidity is due to the presence of H ions in a solution, the acidity increasing as the number of H ions increases. Strong acids are those which are highly dissociated in solution to give a large number of H ions. Weak acids are those which are but slightly dissociated in solution and, therefore, give relatively few H ions.

Buffer Action

"If the solutions which are encountered in chemical processes, bacteriologic work, etc., were only solutions of known pure acids and alkalies, the pH value could be calculated from the titration values and ionization constants. This is, however, seldom true, as the solutions normally contain relatively indefinite quantities of other substances, and usually a number of unknown impurities. Many, and in fact, most of these materials have what is known as 'buffer action,' which is described by Clark as the resistance exhibited by a solution to change in pH through the addition or loss of acid or alkali. This action can best be illustrated by means of an example. Pure water has a pH value of 7.0. If 1 c.c. of 0.01 N HCl is added to a liter of pure water, the pH value will be changed to about 5.0. Let us now consider a solution containing a mixture of sodium acetate and acetic acid. In this solution the dissociation of the acetic acid is very small



The dissociation of sodium acetate is, however, large



Now suppose we add a small quantity of HCl to this solution. It is immediately largely dissociated into H and Cl ions.



"We therefore have in solution HCl, Na and CH_3COO ions. Acetic acid ionizes to only a very slight degree. This means that CH_3COO and H ions cannot exist together in solution to a very large extent. Therefore, the larger part of the H ions from the HCl will immediately combine with CH_3COO ions to form undissociated molecules of acetic acid, and the H ion concentration or pH value of the solution will be only slightly changed, if at all. If, on the other hand, NaOH is added to the mixture, it will react with the H ions to form Na ions and H_2O . More acetic acid will then dissociate into CH_3COO and H ions and the H ion concentration will be practically the same as it was before. It will, therefore, be seen that considerable quantities of acids or alkalies may be added to solutions containing buffer salts without changing the pH value. Since this is true, it is clear that buffered solutions can be diluted with distilled water, even though the water shows a very acid reaction, without affecting the pH

value. In fact, some solutions can be diluted as much as 1,000 to 1. This is of importance in making determinations on very highly colored and turbid solutions.

“In general the salt of any weak acid or weak base is a buffer salt. There are, therefore, very few solutions which are free from buffer action. For example, the phosphates in raw sugar and culture media, carbonates in raw water, alum and resin in paper sizing, etc., have buffer action.

“In order to bring out the importance of making pH determinations rather than determining the total acidity or alkalinity by titration, we shall consider a solution of raw sugar, which contains phosphates as well as other buffer compounds. By the addition of small amounts of acid or alkaline materials, the total acidity or alkalinity is increased and this additional acid or alkali is shown by titration. Since the solution is highly buffered, however, there will be practically no change in the pH value. Since it is the pH of the solution, that is, the amount of ionized acid, which determines the amount of inversion of cane sugar, the reason for determining the pH rather than the total acidity is apparent. This is also clearly shown by the figures given in the introduction. The application is, of course, similar in other processes.

Unbuffered Solutions

“From the above discussion it will be realized that no special precautions are necessary in making pH measurements on buffered solutions, since the addition of considerable amounts of acid or alkali can be made without affecting their pH value.

“Such is not, however, true with distilled water or unbuffered solutions, especially when their pH value is near the neutral point of 7.0. The fact that distilled water is one of the most difficult materials to test for pH is frequently overlooked. This is due to the fact that it is absolutely devoid of any buffering action, and is thus susceptible to change during the test, for example, by the absorption of carbon dioxide, etc.

“Pure distilled water is, of course, free from salts and has a pH value of 7.0 at 18° C. However, the reaction of ordinary distilled water is always acid, because of the absorption of carbon dioxide. Water which has taken up carbon dioxide from the air until equilibrium has been established, will contain about 0.3 per cent of CO₂ by volume, and the calculated pH should be 5.7. In fact, this is the value which is usually found in distilled water which has been freely exposed to pure air. Water from an efficient automatic still, when stored in closed nonsoluble glass vessels, will have a pH of 6.0 to 6.4. If this water is boiled for a short time in a Pyrex vessel, and the vessel then fitted with a soda lime tube, it will usually have a pH of 6.6 to 6.8.

“When it is necessary to make a solution of an unbuffered material in order to determine its pH value, the water used should, of course, be as nearly neutral as possible, as any acidity of the water will affect the pH of the dissolved material. Water having a pH value of 6.6 to 6.8 is satisfactory for this work. In making these solutions, the proportion of material to water should always be kept the same, so that different determinations will be comparable.

It is, of course, equally true that the indicator solutions should have a neutral reaction, since any excess acid or alkali will likewise change the pH value of the material.

“These precautions are particularly important when the pH value of the material is near 7.0. Around this point small variations in hydrogen ion concentration, due to absorption of carbon dioxide, etc., have a marked effect on the pH value. This effect is, of course, much less marked when we get below 6.0 or above 8.0.

“In making a test on distilled water or unbuffered solutions, the indicator and the material being tested should always be mixed in the test tube by means of a stirring rod, with the minimum of exposure to air, and readings should be made at once. If this is done, reliable results will be secured with the colorimetric method. In fact, this is the only way in which such solutions can be tested for pH, since electrometric methods are very unreliable when applied to most unbuffered solutions.”*

Potentiometer Measurement of pH

A very widely used method for measuring pH employs a potentiometer, which measures the voltage developed between a pair of suitable electrodes immersed in the solution. This voltage is translated into the equivalent pH. The method is quick and accurate and is well suited to automatic recording, signaling, and controlling.

Measurement of pH.—The simplest method for determining pH is to measure the difference of potential set up between two electrodes immersed in the solution to be tested. One, the measuring electrode, assumes a potential dependent on the pH value of the solution. The other, the reference electrode, establishes a reference potential. The electrical method can be applied under such seemingly adverse conditions as deep color, turbidity, heavy sludge, minute samples, or large bulk, and is the standard method by which all others are tested, including the colorimetric method.

The electrical measurement is properly made under conditions which involve no appreciable flow of current through the electrode circuit, except momentarily while the measuring apparatus is being adjusted, since flow of current through the electrodes causes two errors. One, which lasts only while the current is passing, is a change in the true electrode potential equal to the product of the current flowing and the resistance of the electrode system. The other, which persists for a time after current ceases to flow, is due to a change of the potential at the electrodes and is known as polarization. These errors may be prohibitive if an improper method of measurement is used.

The null potentiometer meets the conditions for negligible error explicitly, and in addition easily conforms to any required precision. In this instrument the difference of potential to be measured is balanced against a difference of potential derived from a known constant current passing through a known resistance. The balance is obtained by adjusting the tap points of the resistance so that the deflection of a galvanometer in the electrode circuit becomes zero.

*Taylor, W. A.: Publication of La Motte Chemical Co.

Since no appreciable current flows in the electrode circuit at the time of balance, and since the disturbing effect of previous current flow can be limited to a very small magnitude, the conditions for accurate measurement are satisfied.

After the difference of potential is determined, it is converted into the equivalent pH value by reference to suitable equations, tables, or charts. For use with a particular electrode, some instruments are calibrated to read directly in pH. Within a stated limit of error, the value so obtained is the correct pH of the solution at the existing solution temperature.

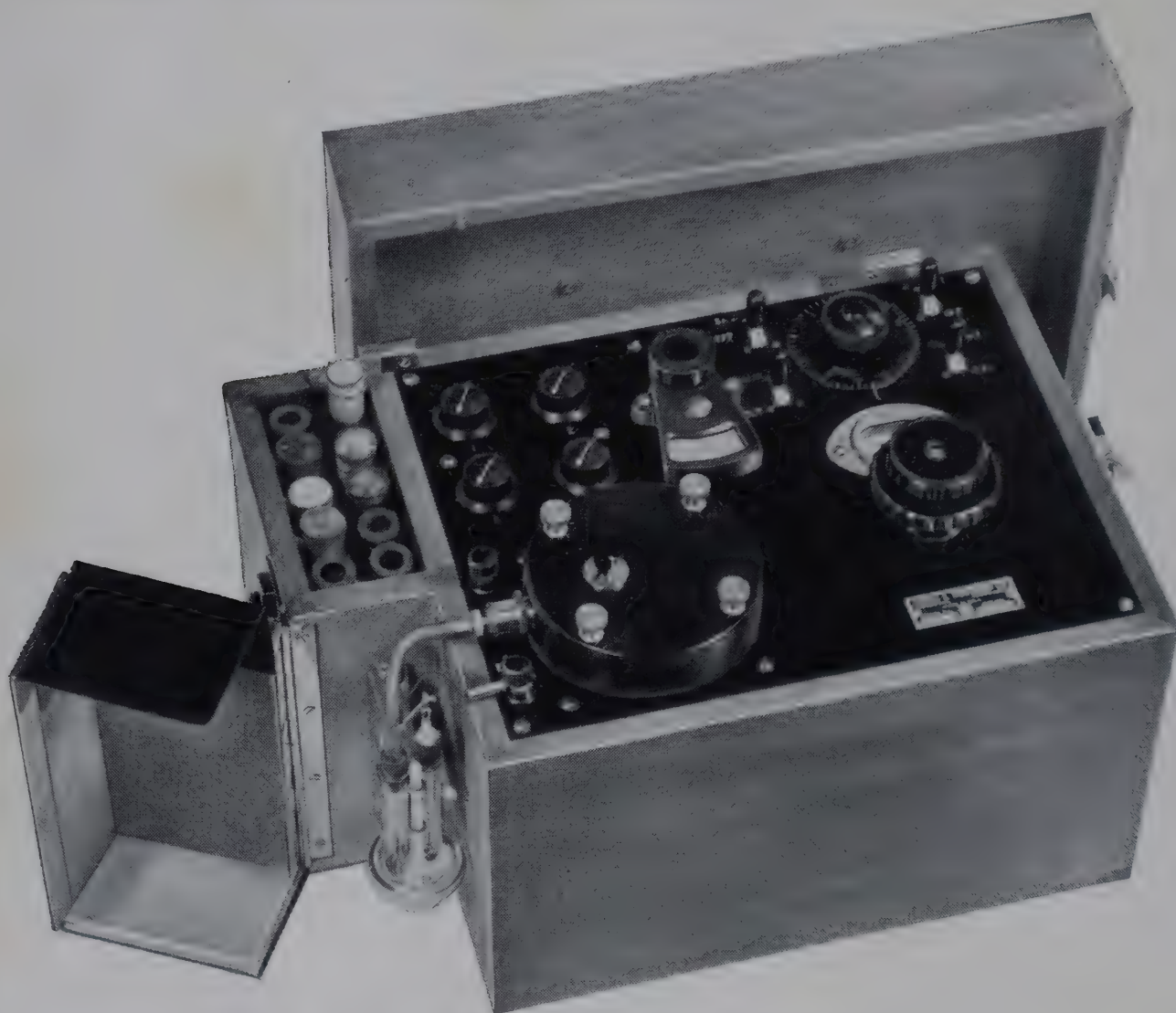


Fig. 13.—Potentiometer. (Leeds & Northrup Co.)

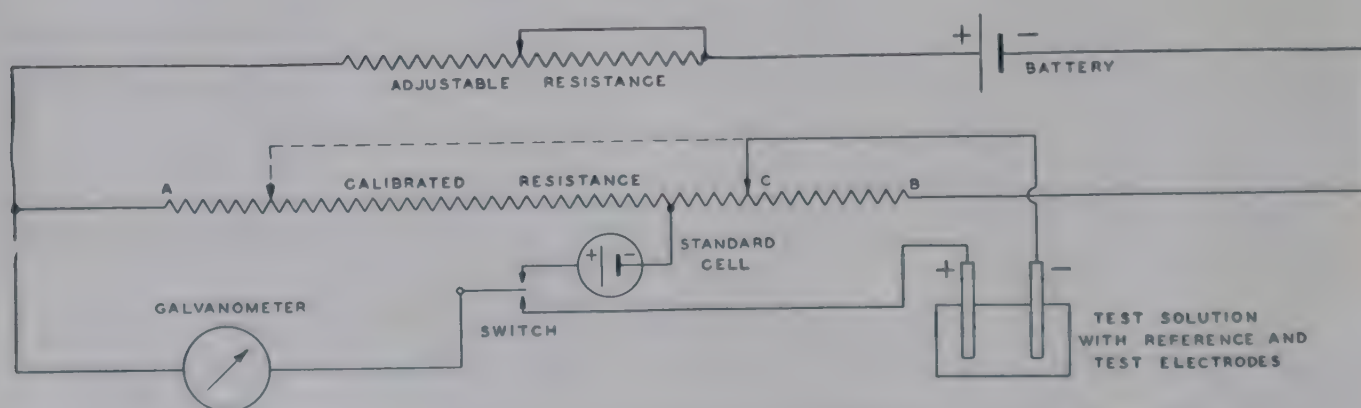


Fig. 14.—Diagrammatic representation of standardization of the potentiometer.

Elements of the Potentiometer.—Fig. 13 shows the essential elements of the potentiometer circuit used for pH measurement, both in instruments for manual operation and in those which are self-balancing.

To standardize the potentiometer, the current through *calibrated resistance* is adjusted to a standard value by turning *switch* to the upper or *standard cell* position and adjusting *galvanometer* deflection to zero by means of *adjustable resistance*, thus balancing the drop in voltage developed in the calibrated resistance with that of the standard cell.

To measure an unknown voltage, the *switch* is turned to the lower or test position and the *galvanometer* is again adjusted to zero deflection, this time by moving *contact C*. The difference of potential existing between the measuring and reference electrodes in the solution is then equal to the drop in voltage between *A* and *C* and is therefore known.

There are various types of potentiometers, many of which are designed for industrial establishments.

The universal pH indicator, made by Leeds & Northrup Co., enables one to read directly in pH with glass, quinhydrone, and hydrogen electrodes, and with any other pH electrode following the Nernst equation. When used with the glass electrode system furnished, simply setting the dial of the temperature compensator to the sample temperature adapts the entire pH scale to measurements at that temperature. This applies also if a quinhydrone electrode is substituted for the glass electrode. Better standardization than can be made with a standardized buffer solution alone is provided by a high-grade Eppley standard cell. For making accurate potential measurements, the standard cell is essential.

The small rugged electrodes are carried within the instrument case, ready to be put in use in the measuring compartment. A diagram of the universal pH indicator is shown in Fig. 14.

Beckman Potentiometer.—

Operating Principle.—The voltage developed at the electrodes is connected in series opposition with a variable voltage from a precision slide wire potentiometer. The differential voltage is amplified to operate a balance-indicating meter and the potentiometer is adjusted until the differential voltage becomes zero. At this condition the voltage of the potentiometer is exactly equal to the electrode voltage, and the pH may be read directly from the calibrated potentiometer dial.

The amplifier used in this instrument is distinguished by two important features: (1) the condition of balance or unbalance is continuously indicated, permitting smooth rapid potentiometer adjustments with complete freedom from the key-tapping annoyance of the ballistic method; (2) the maximum sensitivity of the amplifier occurs at the balance point and the sensitivity readily decreases with increasing unbalance—a safety feature which automatically protects the indicating meter from damage by excessive unbalance and eliminates sensitivity-reducing switches.

In the Beckman pH instrument, the electrode system consists of a factory-sealed glass electrode and saturated calomel electrode. The glass electrode is very sturdy and can be inserted in highly viscous materials without danger of breakage. It requires no maintenance. The calomel electrode requires no maintenance.

Saturated KCl solution in the outer tube makes fluid contact with the test solution through a small hole protected by a rugged ground glass sleeve, effec-

tively preventing contamination. The small electrodes furnished as standard equipment require only 2 to 3 ml. of test solution. Special electrodes are available for quantities as small as 0.005 ml. and for many unusual applications.

The electrode compartment is electrostatically shielded. There is a safety beaker support. The sidewise and vertical motions of the beaker holder are limited to prevent damage to the electrodes from careless manipulation.

This Beckman laboratory model (see Fig. 15) is extremely simple to operate. Temperature is compensated automatically over the entire pH scale for the range 10° to 40° C.



Fig. 15.—Coleman model 20 "Compax" pH meter. (Courtesy Coleman Instruments, Inc., Maywood, Ill.)

Coleman Model 15 Industrial pH Tester.*—

To Assemble.—

1. Unwrap the glass electrode and place in left side of **mounting block** on door, adjacent to the reference electrode. Insert its terminal plug into the large, clear plastic jack located back of "B" knob.

2. The reference electrode and KCl reservoir are shipped assembled and filled, in position on mounting block. Insert the reference terminal plug in black plastic jack.

Calibrating.—

Each morning before use calibrate as follows:

1. Move switch to "T" position.

*Research Department, Coleman Electric Co., Inc., Maywood, Illinois.

2. Wash the glass electrode and liquid junction (rubber tube) with distilled water, wiping the glass electrode clean with paper tissue or a soft cloth.

3. Loosen reference electrode cap one-half turn and make a new liquid junction by squeezing the rubber tube strongly at a point one-half inch above the red tip. (The pressure so generated causes a fine stream of KCl solution to issue from minute orifice in tip and purges the junction.)

4. Fill sample cup half full of distilled water, add to this two "shots" of concentrated 4.1 buffer from white bottle and then place cup on platform with electrodes immersed.

5. With switch still in "T" position adjust meter pointer to red "S" line with "T" knob. Move switch to "B" position and again adjust meter pointer to red "S" line with the "B" knob. (Both of these knobs are located on side of case.)

6. Move switch to "read" and adjust meter pointer to red "S" line with "bal" knob.

7. Move switch to "bal" position and adjust meter to red "S" line with "AP" knob. The instrument is now ready for tests. DO NOT DISTURB "AP" ADJUSTMENT EXCEPT WHEN CALIBRATING.

Measuring Unknown pH.—

1. Rinse sample cup, glass electrode and liquid junction with distilled water. Fill cup half full of sample and place on platform with glass electrode bulb and junction immersed.

2. Move switch to "bal" position and adjust meter pointer to red "S" line with "bal" knob.

3. Release switch and read pH directly from meter scale.

Note: For best accuracy purge the liquid junction and check "B" adjustment after each 6-8 tests as directed above. Check "T" adjustment occasionally. Recalibrate against standard buffer twice daily.

Meter.—

With the switch in the "off" position, the meter should read exactly zero; i.e., the mechanical zero of the meter should fall at zero on the scale. If this is not the case, the mechanical zero of the meter should be adjusted to the scalar zero by means of the screw in the lower center of the meter case. This adjustment should very rarely, if ever, be necessary.

ACID-PROOF TABLE TOPS

This is an excellent method for preparation of laboratory worktables.

Solution No. I.

Aniline hydrochloride	73 grams
Ammonium chloride	40 grams
Water	600 c.c.

Solution No. II.

Copper sulphate	100 grams
Potassium chlorate	50 grams
Water	650 c.c.

By means of a brush apply two coats of Solution No. I while hot, applying the second coat as soon as the first coat is dry.

Then apply two coats of Solution No. II and allow to dry thoroughly. Next, apply a coat of raw linseed oil with a cloth, making this coat as thin as possible.

In the treatment with the oil, the deep black color is only partially brought out. After this oil coat, thoroughly wash with hot soapsuds, to remove all superfluous chemicals, bringing out the full black finish.

CHAPTER II

URINE ANALYSIS

COLLECTING THE SPECIMEN

While it is convenient in most institutions that care for the sick to collect the morning specimen of urine for examination, the examination of specimens collected over twenty-four hours, especially for quantitative tests, is the best method. The urine varies in its composition at different times of the day; a morning specimen may not reflect the same condition seen at noon or at night. If it is not possible to collect the twenty-four-hour specimen, a mixture of the night and morning urine is advisable for examination. There is a difference in the amounts of sugar and albumin in the specimens of urine collected at different times of the day. A specimen taken shortly after a meal is likely to contain more sugar and albumin than one taken the first thing in the morning. The containers in which the urine is collected should be chemically clean and absolutely sterile. In many hospitals regulation urine bottles with labels and stoppers are sent from the laboratory to the different divisions. The specimen should be received either directly into this container or poured into it from the receptacle in which it is received.

Voided specimens of urine from female patients are likely to be contaminated with albumin and pus from vaginal discharges and are also likely to be contaminated with albumin and blood from menstrual discharges. Therefore catheterized specimens are to be preferred for examination and represent the only reliable material for analysis.

URINE PRESERVATIVES

Preservation of urine for chemical and microscopic examination has been the subject of much study by the large insurance companies, to whom this problem is most important. It is equally as important for all those engaged in the laboratory examination of urine because under normal conditions bacterial action quickly produces changes in urine specimens that impair the value of analysis. Urea forms ammonium carbonate, sugar decomposes into carbon dioxide and water, the solution becomes turbid, and the formed elements of the sediment disintegrate. It is obvious that conclusions based on such a specimen would be of little value. In medical practice the urine specimens may deteriorate during the time they stand in the patient's home and in the physician's office before reaching the clinical laboratory for analysis.

Because of the urgent need of a satisfactory preservative the Biochemical Laboratory of the Metropolitan Life Insurance Company made extensive field tests in 1924 and 1925 to determine the best preservative for its purpose and finally developed a formula which has been used by the Metropolitan Life Insurance Company and others in more than two million specimens.

We are indebted to Dr. N. R. Blatherwick, biochemist of the Metropolitan Life Insurance Company, for information regarding preservation of urine. The tablet which he recommends was compounded by Dr. Francis B. Kingsbury.¹ Its composition is as follows:*

	PER TABLET FOR 1 OUNCE URINE
Potassium acid phosphate	0.100 gm.
Sodium benzoate	0.050 gm.
Benzoic acid	0.065 gm.
Urotropin	0.050 gm.
Sodium bicarbonate	0.010 gm.
Mercuric oxide, red	0.001 gm.
	0.276 gm.

This tablet does not interfere with the quantitative determination of albumin by the sulphosalicylic acid method nor with the determination of sugar by Benedict's picate method. Casts, red blood cells, and leukocytes are well preserved. Experience has shown that one tablet will preserve up to two ounces of urine.

Additional information on the preservation of urine was obtained from Dr. R. H. Feldt, assistant medical director of the Northwestern Mutual Life Insurance Company, Milwaukee, Wis. He recommends the Kingsbury tablet (boric acid).

Prepare containers by fractional sterilization, putting 10 grains of boric acid into each two-ounce container. Urine voided under reasonably careful precautions in a clean vessel and transferred to this container will not decompose within three to seven days. Boric acid will not, however, prevent further growth of organisms in urine contaminated before being put into the container. The boric acid is easy to handle in the form of 5 gr. tablets, does not produce false copper reactions in reduction tests, and does not interfere with fermentation in the quantitative determination of sugar. Chloral hydrate, sometimes used as a urine preservative, under certain conditions reduces copper solutions. Formaldehyde offers the same objections. Either formaldehyde or toluol may at times cause a false ring with Heller's test.

EXAMINATION

Volume.—Volume in twenty-four hours is measured by pouring the urine into a 1,000 c.c. cylinder, and reading the number of cubic centimeters present. The normal volume of urine in 24 hours is 750 to 2,000 c.c., or 25 to 65 ounces. The average is 1,500 c.c.

Color.—Record the color of the urine; for example, "very pale yellow," "light amber," "dark amber," "greenish," "brown." The color of the urine is dependent upon many factors. Normally, it is yellow, or reddish yellow, due to the presence of several pigments, especially urochrome. The intensity of the color depends upon the concentration of the urine. Dilute urines are usually pale, while concentrated urines are darker. Acid urines are usually darker than alkaline urines. The color may be greatly changed by the presence of abnormal pigments and by various drugs as follows:

Blood: red or brown; smoky

Bile: yellowish or brown, turning greenish; yellow foam.

¹Proc. A. Life Insurance Med. Directors 13: 104, 1925-26.

*These tablets may be obtained from Fales Chemical Company, Cornwall Landing, New York. They are also available from Cargille Scientific, Inc., 118 Liberty St., New York City 6.

Chyle: milky.

Methylene blue: greenish blue.

Phenols: olive green to brownish black, etc.

The color of urine is often affected by drugs.—

Dark green: phenol, salol, creosote, resorcinol

Red: antipyrine, trional

Brown or black: pyrogallol

Yellow: phenacetin, picric acid. Santonin changes from bright yellow to scarlet with the addition of sodium hydroxide.

Red urine is also caused by eating beets.

Odor.—Record as “normal,” “aromatic,” “medicated,” “ammoniacal.” “putrid,” “strong.”

Transparency.—Record as “clear,” “cloudy,” “turbid,” “cloudy with sediment.”

Freshly voided normal urine is clear or transparent, but may be cloudy as a result of the presence of phosphates and pus. The phosphates disappear upon the addition of acid; pus does not disappear, but may become gelatinous. A freshly voided urine may be cloudy from the presence of bacteria, or comparatively clear with numerous shreds of mucopurulent material from chronic urethritis.

The transparency record is a factor only in dealing with fresh specimens, because all specimens become cloudy with bacterial and alkaline salt formations upon standing.

On cooling and standing, all specimens develop a faint cloud of mucus, leukocytes, and epithelial cells which settle to the bottom—the so-called “nubecula.” Acid urines may develop a white or pinkish sediment of amorphous urates, while alkaline urines may develop a heavy white sediment of amorphous phosphates. Urine containing pus shows a heavy mucoid, whitish sediment. Blood gives a reddish brown smoky sediment. Uniform cloudiness which cannot be removed by ordinary paper filtration is due to the presence of bacteria. Turbidity of urine is often due to the presence of fat or of spermatozoa.

Reaction

Dip a piece of blue litmus paper into the urine. If it turns pink, the urine is acid. If it does not turn color, place a piece of red litmus paper in the urine. If it turns blue, the urine is alkaline. If neither the blue nor the red paper turns, the urine is neutral. If the red litmus turns blue and the blue litmus turns red, the reaction is amphoteric.

Normal urine is acid in reaction with a pH ranging from 4.8 to 7.5, or a general average of 6.0. Urine may be neutral or alkaline as a result of the administration of alkalies. Diet may influence the reaction. Acid urine is seen in acidosis, diabetes, starvation, diarrhea, etc. Alkaline urine occurs in alkalosis, severe vomiting, urinary infections, etc.

Determination of Hydrogen Ion Concentration.—The hydrogen ion concentration is determined with the indicator solutions. For this method, use fresh clear urine. Use the method outlined on pages 30 and 31, and also see Potentiometer measurement of the pH, on pages 35 ff.

Nitrazine Indicator for Use in Acidimetry and Alkalimetry.—

Nitrazine* is a very sensitive indicator for use in acidimetry and alkalimetry, both for titration purposes and as an indicator of hydrogen ion concentration. It is especially useful in determining the reaction of urine, saliva, and other body fluids. In cardiorenal disease, the mean acidity can be about pH 5.3. The composition of food is perhaps the most important factor in determining the reaction of urine. The so-called alkaline tide occurs shortly after eating, because of the increased alkalinity of the body fluids subsequent to the loss of acid for purposes of digestion.

Urine that has stood for some time becomes alkaline to litmus as a result of fermentation due to bacteria. The alkaline reaction of urine on a vegetable diet is due to the fact that vegetables and fruits, including acid fruits, contain acid salts of dibasic or other organic acids. These acids are burned in the body to carbonates and have an alkaline reaction because they form some free alkali by hydrolysis. Part of the carbonic acid is lost to the body through the lungs, leaving the associated strong base together with some of the very weak carbonic acid to find their way into the urine.

Alkalinity of the urine can be raised by the ingestion of sodium bicarbonate.

This indicator, nitrazine, changes color within approximately the same range as litmus, but, since the indicator is more sensitive and the color changes are much more definite, differential readings can be made over smaller pH intervals.

Another advantage of nitrazine is that the readings can be made and must be made within a minute or two after applying a drop of the solution to the paper. When using nitrazine test solution the reaction is immediate and permanent.

The color values on nitrazine paper observed when read in from 60 to 120 seconds, with the solution applied with glass rod, are as follows:

pH 4.0 bright yellow	pH 6.4 slightly stronger bluish cast to olive green
pH 4.5 yellow, slightly duller (lemon yellow)	pH 6.5 dark green
pH 5.0 mustard yellow	pH 6.6 blue gray
pH 5.3 light olive yellow	pH 6.8 dark blue gray
pH 5.5 olive yellow	pH 7.0 gray blue
pH 5.7 olive green with yellow cast	pH 7.3 deeper blue
pH 6.0 olive green	pH 7.6 same
pH 6.2 bluish cast to olive green	pH 8.0 same

Nitrazine is available in two forms:

- (1) In paper strips, 100 to a vial, 10 vials to a box.
- (2) As a 0.1 per cent solution in dilute alcohol in a 4 oz. bottle.

Hydrion pH Test Papers†

Under the trade name Hydrion Wide-Range pH Test Papers, there is available another method of determination of reactions. There are five types of Hydrion paper, showing changes at the following pH values: Hydrion A, pH 2-4-6-8-10; Hydrion B, pH 1-3-5-7-9-11; Hydrion C, 11-12; Hydrion D, 12-13; and Hydrion E, 13-14. These papers are dispensed in rolls in a special machine for turning out a piece of the paper when its use is indicated. The paper is also furnished in strips in a vial.

Hydrion pH buffers are also supplied in vials. Each buffer when dissolved in 100 c.c. of distilled water gives a solution of the indicated pH. The manufacturer supplies the complete set of 55 standards in a permanent wooden case, pH 1.2 to 12.0 in steps of 0.2 pH unit.

*Manufactured by E. R. Squibb & Sons.

†Hydrion reagents are manufactured by Micro Essential Laboratory, Brooklyn, New York, and may be procured from Cargille Scientific Inc., 118 Liberty St., New York 6, N. Y.

Anachemia Papers

The Anachemia, Ltd., of Montreal, Canada, makes a variety of test papers in a wide range type from pH 1.0 to pH 12.0 as well as an acid range of pH 0.0 to pH 5.0 and alkaline to pH 14.0. In addition, twenty fractional range types are made, thus making available an indicator allowing accuracy to within 0.3 pH unit to 0.1 pH unit by use of overlapping papers.

Titratable Acidity of Urine in Terms of N/10 Sodium Hydroxide.—

Folin's Method

Place in a beaker 10 c.c. urine.

Add 6 grams of finely pulverized neutral potassium oxalate and 1 to 2 drops of phenolphthalein indicator.

Shake the mixture vigorously for one to two minutes.

Titrate with N/10 sodium hydroxide solution from a buret, until the solution turns a faint permanent pink.

Read from the buret the number of c.c. of N/10 sodium hydroxide used.

Calculation:

Assume the volume of the 24-hour specimen is 1200 c.c.

The number c.c. of N/10 sodium hydroxide used is 6.

10 c.c. urine used in the test.

$10 : 6 :: 1200 : x$

$10 x = 7200.$

$x = 720$, the acidity of the twenty-four-hour specimen in terms of N/10 sodium hydroxide.

To determine the percentage of acidity, obtain the degree per 100 c.c. of urine. In the example above, 720 would be divided by 12, as there are 1200 c.c. of urine in the twenty-four-hour specimen. The percentage in this case is, therefore, 60.

Specific Gravity

Consult pages 165 ff. for concentration and dilution tests, which utilize specific gravity determinations.

Pour the urine into a cylinder. Place a urinometer in the urine, spinning to prevent its floating to the side. Read the specific gravity from the stem of the urinometer.

The urinometer is always calibrated for use at a certain temperature. If the specific gravity is taken at any other temperature, one unit of the last order must be added to the reading for every three degrees above the normal temperature, and subtracted for every three degrees below the normal temperature.

Example.—The urinometer is calibrated for a temperature of 15° C. The specific gravity of the urine at 18° C. was 1.022. True specific gravity is $1.022 + 0.001$, or 1.023.

The normal specific gravity is from 1.015 to 1.020. In diseased conditions, it may vary from 1.001 to 1.060. The Exton immiscible balance supplied by Emil Greiner Company of New York is useful to determine the specific gravity of drops of urine when only very small amounts are available, as in ureteral catheterization. The method and construction of the instrument are based on the principle of suspending the urine in an immiscible medium of the same specific gravity which is then determined by the usual methods, making possible rapid

manipulations with minimal loss of material. A cylindrical mixing chamber is partially filled with a mixture of petroleum ether and carbon tetrachloride with a specific gravity of about 1.012. The side thistle tube is filled with petroleum ether and the carbon tetrachloride is kept at hand in a drop bottle. A drop of urine is immersed in the mixture, which is then easily and rapidly varied by means of the stopcock and dropper, so that the urine remains suspended near the middle. The specific gravity of the mixture is then taken by means of the Exton hydrometer; this gives the specific gravity of the urine.

Solids

The amount of solids in 1,000 c.c. may be roughly calculated by means of Long's coefficient, which is 2.6. Multiply the last two figures of the specific gravity at 15° C. by 2.6 to obtain the solids.

Example.—

Specific gravity at 15° C. was 1.016.

$16 \times 2.6 = 41.6$ grams in 1,000 c.c. urine.

The output of a normal adult is about 60 grams. It must be remembered that the output of urinary solids is influenced by body weight, diet, exercise, age, metabolism, and state of kidney function.

SUGAR IN URINE

Grape sugar, or dextrose, exists in every normal urine in small quantity. Moritz¹ states that it can be found in the percentage of 0.05 normally. We assume, however, in our ordinary laboratory tests, that the presence of sugar in the urine is a pathologic condition, or the result of disturbed physiology.

Dextrose, or grape sugar, has the formula:



At times we find in the urine other monosaccharides, such as levulose, pentose, and galactose. We are also interested in certain disaccharides which consist of one molecule of grape sugar and one molecule of fruit sugar; for example, lactose, which consists of one molecule of dextrose and one molecule of galactose. Different sugars in urine have different clinical significance.

A large sugar content in urine also means a high specific gravity, even though the color of the urine may be light and the daily output increased.

Pathologically, we find sugar present in the urine in undue quantity. This condition is called glycosuria. It is a common incident in the disease known as diabetes mellitus. Glycosuria also is found in other diseases; for instance, in connection with certain poisons, such as morphine, carbon monoxide, mercuric chloride, etc. After prolonged hunger, dextrose has been found in the urine.

Alimentary Glycosuria.—

Alimentary glycosuria is a condition caused by undue ingestion of sugar and other carbohydrates by healthy individuals. Excessive ingestion of dextrose will so overload the blood stream with blood sugar that the threshold point will be rapidly reached.

¹Moritz: Deutsche Arch. f. klin. Med. No. 46, p. 217.

Renal Glycosuria.—

Renal glycosuria has at times been called renal diabetes but the proper name is renal glycosuria. In this condition there is usually a pathologic amount of sugar in the urine without any corresponding rise in blood sugar such as is seen in diabetes mellitus. Likewise, there are no apparent symptoms of diabetes mellitus. It is due to the lowering of the threshold point. This condition is supposed to be rare but recent studies refute this impression or at least bring its validity into question. It is benign and carries a good prognosis. The reports of Blotner and Hyde¹ and the Peels² regard renal glycosuria as a relatively common finding, the former reporting one person in 11 with melituria as having the disorder. Joslin and his associates,³ in a review of 18,000 cases of melituria, report 53 cases of renal glycosuria per se and 9 cases of renal glycosuria of pregnancy. Wilder⁴ has reported 82 cases from the Mayo Clinic and Fowler⁵ noted 7 cases of renal glycosuria in 4,000 cases of melituria at the Montreal General Hospital. Marble³ has set the following criteria for the diagnosis. These standards should be fulfilled prior to making the diagnosis.

1. Fasting blood sugar is within normal limits and a normal glucose tolerance curve.

2. Glucose should be present in appreciable quantity in all urine specimens, whether voided in the fasting state or post-prandially. The quantity of sugar in the urine should be for the most part independent of the diet.

3. Carbohydrate utilization should be normal as evidenced by the respiratory quotient and serum inorganic phosphate after glucose ingestion.

4. Fat metabolism should be normal, ketosis being more likely to develop when the patient fasts than when he overeats.

5. Moderate doses of insulin should have little or no effect on the glycosuria.

Bland⁶ reviewed the literature and reported four cases, all of which conformed in every particular. He called attention to the fact that many people with melituria of some type are treated for diabetes mellitus and that real damage may be done by such treatment. Since these patients are losing sugar steadily in the urine, it would seem only sensible that a liberal carbohydrate diet is indicated. Certainly, insulin treatment can be of no value in the treatment of renal glycosuria and may do considerable harm. These patients frequently complain of ease of fatigue and malaise, which symptoms may well have their origin in a low carbohydrate intake.

Renal glycosuria is characterized by a low renal threshold for sugar. Urinary sugar is present because of incomplete reabsorption of sugar by the proximal convoluted tubules of the kidneys. The reason for this abnormality is not well understood. All of Bland's cases had low renal thresholds as

¹Blotner, H., and Hyde, R. W.: *J. A. M. A.* **121**: 432, 1943.

²Peel, A. A. F., and Peel, M. W.: *Glasgow M. J.* **135**: 141, 1941.

³Joslin, E. P., Root, H. F., White, P., and Marble, A.: *The Treatment of Diabetes Mellitus*, ed. 7, Philadelphia, 1940, Lea & Febiger, p. 714.

⁴Wilder, R. M.: *Clinical Diabetes Mellitus and Hyperinsulinism*, Philadelphia and London, 1940, W. B. Saunders Co., p. 28.

⁵Fowler, A. F.: *Ann. Int. Med.* **7**: 518, 1933.

⁶Bland, J. H.: *Ann. Int. Med.* **29**: 461, 1948.

proved by the presence of glycosuria with a normal or subnormal level of blood sugar. It has been postulated that a deficiency of tissue phosphatase may be the abnormality producing consequent interference with the phosphorylation mechanism in the renal tubules on which sugar reabsorption depends. Another possible etiology was that suggested by Thomas and Southworth,⁷ who conjectured that the renal threshold is under hormone control. Comparison is made with diabetes insipidus in which the hormone of the posterior pituitary exerts its well-known effect on the renal tubules. The renal glycosuria of pregnancy fits this conception in that there exists in pregnancy a hormonal imbalance. In Bland's second case, the renal glycosuria developed after a series of streptococcal infections. He suggested that this may be more than coincidental. The widespread vascular lesions apparently produced by repeated hemolytic streptococcal infections may well produce changes in the blood supply to the renal tubules, which, in turn, could alter the tubular physiologic function.

In reference to treatment, a liberal and varied diet should be given with perhaps some emphasis on carbohydrate intake. The patient should be advised that his condition is benign and be reassured that his prognosis is good. Insulin is certainly not indicated. Somogyi⁸ has shown that a low carbohydrate intake and ill-advised use of insulin may produce increased hepatic glycogenolysis with each episode of hypoglycemia, thus leading to increased hepatic fat and protein metabolism, with consequent increase in ketone production. Thus, a clinical ketonuria may be induced in the absence of glycosuria. In such an instance, insulin is clearly contraindicated.

Bland's study included determinations of both arterial and venous blood sugars in the glucose tolerance tests in order to prove adequate removal of glucose by the tissues from the arterial blood. Serum phosphorus determinations were simultaneously made to study the absorption of the glucose and the phosphorylation mechanism.

Pentosuria.—

So far as other reducing substances are concerned, pentosuria must be considered. In this condition there is a constant excretion of pentose in the urine regardless of the diet. In pentosuria it is believed that there is a disturbance in metabolism and that it is not a precursor of diabetes mellitus. It is essential to differentiate pentosuria from glycosuria because the pentosuric patient must not be subjected to the rigid dietetic control or insulin treatment of the diabetic patient. Tests to be described later (page 57) serve to differentiate pentosuria from glycosuria and levulosuria.

An abundance of levulose or fructose is noted at times. These cases of levulosuria or fructosuria are seen after the ingestion of large amounts of fruits. In other words, it is an alimentary condition rather than a metabolic condition. Tests later to be described serve to differentiate levulose from dextrose. The Seliwanoff test on page 56 is the best test for this purpose.

⁷Thomas, H. M., Jr., and Southworth, H.: *Ann. Int. Med.*, **12**: 1560, 1939.

⁸Somogyi, M.: *J. Biol. Chem.* **141**: 219, 1941.

Alkaptonuria.—

Alkaptonuria is a condition due to the presence of homogentisic acid in the urine. In this condition, the urine becomes dark after prolonged standing. This is due to oxidation processes. The urine becomes dark brown to brown black in color. One can see on the patient's contaminated clothing dark red or brown spots where the condition exists. It is seen frequently in small children. The addition of 10 per cent sodium hydroxide results in a brownish-black ring at the surface which gradually penetrates downward. Ferric chloride causes a transitory blue color with each drop, and reduction of silver lactate and of ammoniacal silver nitrate proceeds rapidly at room temperature. With Millon's reagent, a lemon yellow precipitate occurs. With heat, this precipitate becomes red. The ammoniacal silver nitrate solution results in a production of a black color when added to alkaptonuric urine.

Millon's Reagent.—Metallic mercury, 1 part. Fuming nitric acid, specific gravity, 1.420, two parts. Dilute with an equal part of water and after a few hours, decant.

Ammoniacal Silver Solution.—Silver nitrate, 26 gm. dissolved in 200 c.c. of water. Add ammonia. A brown precipitate will fall; add more ammonia until this precipitate is redissolved. Make the volume up to one liter with distilled water.

This condition is evidently an anomaly of protein metabolism and is probably inherited. It appears to be a rare recessive character in the Mendelian sense. Its mode of incidence is remarkably similar to that of albinism but its presence is not as strikingly apparent as is that condition. This error in metabolism is present from birth and persists throughout life. An interesting record of two cases in a Negro family was reported by Abbott.¹ This record showed there were six children of a healthy father and mother. One was dead at the time of observation. One brother of thirteen years was alkaptonuric and one girl of eight was alkaptonuric. Another brother and sister were healthy. That they were alkaptonuric since infancy was established by the fact that the father well recalled the staining of the bedclothes by the urine of these two children.

Tests for Sugar in Urine

The determination of sugar in the urine depends upon certain reactions. First, we shall consider the reduction tests. These are based upon the principle that all monosaccharides reduce in alkaline solution of metallic oxides through combination of the acid radical of the aldehyde. Trommer's, Benedict's, and Fehling's tests are based upon this principle.

Another method of reduction is the fermentation test which is based upon the property of yeast plants to break grape sugar into alcohol and gaseous carbon dioxide.

Another method of determination of sugar is through the physical activity of grape sugar, as manifested by the deviation of polarized light.

Polariscope Test

Collect a twenty-four-hour specimen of urine. The reaction must be acid. Add about 2 gm. of finely powdered normal, or neutral, lead acetate; shake vigorously and filter. Return cloudy portions of the filtrate to the filter until the re-

¹Abbott, L. D. F., Jr.: Science 94: 365, 1941.

sulting fluid is clear. Practically no sugar will be held back by normal lead acetate. Fill the polariscope until there is a convex meniscus on the end of the tube. Slip the glass disk in place, taking care to prevent the entrance of any bubbles. Secure the disk in place with a metal cap. Place the tube in the polariscope and examine it with a sodium flame in a dark room. Focus the apparatus. Determine the zero point without the urine specimen, refocus, place the tube of urine directly in the polariscope, start at zero and rotate the handle until the entire field is equally illuminated. The percentage is read directly on the scale of the polariscope. An average of at least six readings should be taken. The percentage of glucose can be read to within about $\frac{1}{10}$ per cent error.

Sources of error:

1. Albumin, which is levorotatory, will counterbalance the glucose and for that reason must be removed from the urine before the test is made.

2. Alkaline urine cannot be used as dextrose in alkaline media may be converted into levulose.

3. If β -oxybutyric acid is present, it will interfere with the estimation of glucose because it is levorotatory.

4. Combined glyconurias are levorotatory but do not interfere with the reaction.

5. Levulose is levorotatory and therefore lowers the glucose reading.

6. Maltose, which is powerfully dextrorotatory, will give urine a very high sugar value. Therefore, where maltose is present, a polariscope should not be used for sugar determination.

Differentiation of Sugars Found in Urine

1. Fermentation is a quality of glucose and fructose.

2. Typical crystalline form of osazone of glucose or fructose differentiates these sugars from lactose.

3. To distinguish between glucose and fructose, Seliwanoff's test (see page 56) is used, or a polariscopic examination may be made.

4. The presence of pentoses is indicated by a positive reaction with Bial's reagent (see page 57).

Qualitative Tests for Glucose in Urine* (Reduction Tests)

Benedict's Method

Reagents.—

Benedict's Qualitative Solution.—

(a) Dissolve 17.3 grams of copper sulphate, c. p., in 100 c.c. distilled water with heat in a small Pyrex beaker.

(b) In a 1,000 c.c. Pyrex beaker, dissolve 173 grams of sodium citrate, c. p., and 100 grams anhydrous sodium carbonate, c. p. (if crystalline sodium carbonate is used, 200 grams are used in place of 100 grams of the anhydrous), in about 600 c.c. distilled water, with heat.

*"In two- to four-plus specimens a yellow film usually adheres to the inside of the tube and defies removal by soap, brush, or cleaner. It will disappear as if by magic if you drop a pinch of dry bicarbonate of soda into the wet tube, moisten the test tube brush and swish it around in the tube, add a little water, and swish it several times more, rinse, and dry. . . ."
Medical Economics. Cleaning of Test Tubes Used in Sugar Analysis.

(c) Filter solution (b). Add the copper sulphate solution slowly and with constant stirring to the mixture of sodium citrate and sodium carbonate, until all has been added. Rinse the residue in the small beaker into the solution, using a few c.c. of distilled water. Cool and dilute to 1,000 c.c. with distilled water.

Technic.—

Place 5 c.c. Benedict's qualitative solution in a test tube. Add 8 to 10 drops of urine and mix. Place the test tube in a cup containing equal parts of boiling water and calcium chloride. Allow the contents of the test tube to boil for two minutes. Let stand until cool.

If glucose is present in the urine, there will be a brick red precipitate. If glucose is present in a very large amount, the entire contents will assume a brick red color. Very small quantities of sugar, as low as 0.015 to 0.020%, will yield a precipitate. Phosphates yield a white precipitate. The degree of reaction is judged by the amount of precipitate.

Benedict's solution, while less susceptible than Fehling's to reduction by other urinary constituents, such as uric acid or creatinine, is, however, promptly reduced by alkaptonic acids or the conjugated glycuronic acids, or by an excess of preservatives such as chloroform, chloral, and formaldehyde. When the urine of a patient shows reduction in the first examination, use further tests to determine whether the substance is sugar, and the kind of sugar.

Streptomycin, in concentrations as low as 200 micrograms per c.c., reduces alkaline copper reagents, such as Benedict's solution. With drug levels greater than 700 micrograms per c.c., it is possible that the reaction obtained with Benedict's reagent may be interpreted incorrectly as a positive test for glycosuria.¹

Unless the urine contains enough reducing substance to reduce Benedict's solution in the heating time specified, it is not clinically significant. Remove the protein from the urine by acidifying with acetic acid, boiling, and filtering.²

Fehling's Method

Reagents.—

Fehling's Solution No. I.—Copper sulphate, crystallized, c. p., 34.639 grams, dissolved in about 300 c.c. distilled water with gentle heat. Dilute to 500 c.c. with distilled water.

Fehling's Solution No. II.—Dissolve 173 grams potassium and sodium tartrate, c. p., crystallized, and 100 grams potassium hydroxide, c. p., in about 300 c.c. distilled water, and dilute to 500 c.c. with distilled water.

Technic.—

Mix equal parts of solutions Nos. I and II in a test tube. Dilute this with two or three times the amount of water, and boil for a few seconds.

If the mixture remains clear after boiling, the solutions may be used. Continue the boiling, adding the urine to be tested, drop by drop. If sugar is present, a yellow precipitate will form. If sugar is present in large amount, the first few drops will yield a precipitate. If the quantity of sugar is small, urine is added until an equal volume has been used. If no precipitate forms, the specimen contains no sugar.

Nylander's Method

Reagent.—

Dissolve 5 grams Rochelle salts (sodium and potassium tartrate) in 100 c.c. of a 10% solution of sodium or potassium hydroxide. Warm, and add 2 grams bismuth subnitrate. Cool and filter through filter paper. Keep in a dark bottle.

Technic.—

Place 5 c.c. urine in a test tube.

Add 0.5 c.c. Nylander's reagent.

Heat for three to five minutes.

Allow to stand for a few moments before reading.

¹The Story of Streptomycin; Merck and Co., Inc., 1946.

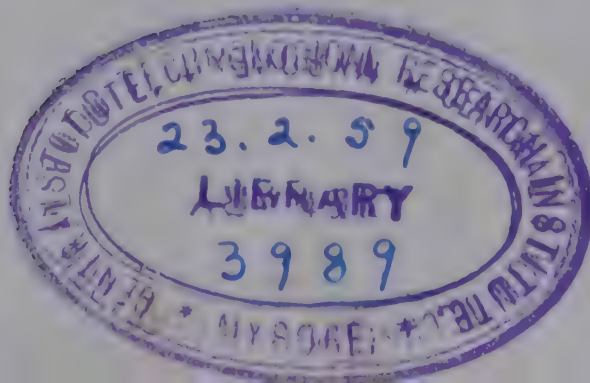
²J.A.M.A., p. 2220, June, 1934.



PLATE II.—BENEDICT TEST FOR SUGAR.

1. Green—Showing Only a Trace of Sugar.
2. Red—Showing a Large Amount of Sugar.
3. Yellow—Showing a Small Amount of Sugar.

(Gradwohl and Blaivas.)



A black color indicates the presence of sugar. A brown color is indicative of a trace of sugar. If the solution turns black after cooling, the reaction is due to substances other than sugar. Phosphates yield a white precipitate. If albumin is present in large amount, remove it by boiling and filtration, since it gives the same reaction as sugar.

Moore-Heller's Method

Place in a test tube 2 parts of urine and 1 part of a 10% potassium hydroxide solution. Boil the upper portion for two or three minutes. If phosphates are precipitated in large amount, remove by filtration.

If sugar is present the following reactions take place:

1% or less of sugar yields a canary yellow color

1 to 2% gives a wine yellow color

2 to 3% gives a sherry color

3 to 4% gives a rum color

Above 4% gives a dark brown or black color

Phenylhydrazin Test for Sugar

Place 10 c.c. urine in a test tube.

Add 0.4 gram phenylhydrazin hydrochloride
and 0.8 gram sodium acetate.

Place the test tube in water-bath and boil for $\frac{1}{2}$ to 1 hour. Allow it to cool. Within half an hour, typical crystals of phenylglucosazon separate when sugar is present, which appear microscopically as fine, bright yellow needles, arranged in bundles, sheaves, or rosettes.

Quantitative Estimation of Glucose in Urine

Somogyi's Method

Somogyi¹ has approached the question of carbohydrate utilization or tolerance of diabetic patients along the line suggested by Naunyn and his school at the turn of the century. It is a method of preparing daily balance sheets of carbohydrate consumption and loss of glucose in the urine, the balance representing the amount of carbohydrate utilized in twenty-four hours. In cases in which insulin therapy is applied, Somogyi advises estimating the total glucose output for twenty-four-hour periods, running separate quantitative determinations on every sample of urine collected between insulin injections. The information thus obtained proved to be the best criterion for the adequacy of the insulin dosage. One of the advantages of this procedure is that it can be pursued for any length of time without hardship to the patient, whereas gathering of the equivalent information by serial blood sugar determinations is scarcely tolerable for days and nights in a row.

The basis of this method is the well-known change of color which takes place when sugars are heated in alkaline solution; the shade which develops in the reaction varies from pale yellow to dark amber, depending upon the concentration of sugar. Sodium carbonate is the alkali which satisfies the requirements of this test. Glucose quantities varying from 1 to 30 mg. in 5 to 6 c.c. of reaction mixture give maximum color intensities when the concentration of the carbonate varies within the wide range of 8 to 15 per cent. The maximum color appears after heating alkaline glucose solutions in a boiling water bath for seven minutes; prolongation of the heating beyond ten minutes

¹Somogyi, Michael: J. Lab. & Clin. Med. 26: 1220-1223, 1941.

causes a gradual fading of the color. Boiling over an open flame for one and one-half to two minutes produces the same colors as heating in the water bath for seven to ten minutes.

Reagents.—

10% Anhydrous Sodium Carbonate.—

Dissolve 10 grams anhydrous sodium carbonate in about 50 c.c. distilled water, and dilute to 100 c.c. with distilled water.

Equipment.—

Test tubes of 14 mm. inside diameter. All test tubes employed must be of identical diameter. A simple way to select uniform test tubes is to measure accurately 10 or 15 c.c. portions of water into a series of tubes and to retain for use only those in which the water level is of the same height.

Pipettes for measuring 0.5 c.c. and 5.0 c.c. portions of fluid.

Technic.—

Introduce into test tubes 0.5 c.c. portions of the urines to be analyzed, then add to every test tube 5 c.c. of the sodium carbonate reagent. Mix the contents, place the test tubes in a rack, and heat, immersed in boiling water, for eight minutes. Racks accommodating from one to two dozen (or more) test tubes are serviceable.

After eight minutes' heating read the results by matching the colors against the standards in the comparator block.*

If the color of the unknown does not closely match any one of the standards, but lies between two of them, estimate the intermediate value by interpolation. If, for example, the color shade of an unknown is halfway between the 3.0 and 4.0 per cent standards, its sugar content is 3.5 per cent; if it is between 3.0 and 4.0 per cent, but distinctly nearer the 3.0 per cent standard, then it contains from 3.2 to 3.3 per cent of glucose, etc.

A source of error because of fading of the standards has been eliminated by Somogyi by devising permanent standards. The stock solution for permanent standards is a 0.01 normal iodine solution in absolute alcohol from which a series of solutions is prepared by dilution with absolute alcohol. The normality of the several diluted solutions is given in Table 4.1 An 0.008 normal alcoholic iodine solution is of the same color, shade, and intensity as the 6 per cent glucose standard and thus takes the place of it; a 0.0058 normal iodine solution is a substitute for a 5 per cent glucose standard, and so on, as given in Table 4.

TABLE 4. CONCENTRATIONS OF ALCOHOLIC IODINE SOLUTIONS USED AS PERMANENT STANDARDS

NORMALITY OF IODINE SOLUTIONS	CORRESPONDING PERCENTUAL GLUCOSE CONCENTRATIONS
0.0004	0.5
0.0009	1.0
0.0018	2.0
0.0028	3.0
0.0043	4.0
0.0058	5.0
0.0080	6.0

In Table 5, one can see the results of the Somogyi method with true sugar values estimated by a copper-iodometric method.

Approximately 5 c.c. portions of these iodine solutions are introduced into Pyrex test tubes of 14 mm. inside bore and the tubes are then sealed. In this condition the standards remain unchanged without requiring any special precautions, as, for example, protection from light or changes in room tempera-

*Sets of permanent standards with a matching block were placed on the market by A. S. Aloe Co., St. Louis, Mo., and are available through laboratory supply houses.
1Somogyi: *Loc. cit.*

TABLE 5.¹ COMPARISON OF RESULTS OF URINARY SUGAR DETERMINATIONS BY THE SOMOGYI METHOD, WITH TRUE SUGAR VALUES ESTIMATED BY A COPPER-IODOMETRIC METHOD

NO.	GM. OF SUGAR PER 100 C.C. OF URINE	
	COPPER-IODOMETRIC METHOD, WITH SHAFFER-SOMOGYI REAGENT NO. 50	SOMOGYI METHOD
1	0.21	0
2	0.42	0.3
3	0.31	0.5
4	0.41	0.3
5	1.2	1.2
6	1.3	1.3
7	2.1	2.0
8	2.6	2.5
9	3.0	3.0
10	3.9	4.0
11	5.0	5.0
12	5.0	5.0

tures. The permanence can be readily ascertained by comparison with standards freshly prepared with pure glucose solutions. The earliest permanent standards in Somogyi's laboratory after two years showed no deterioration.

Somogyi's experience, which is very wide, in using these permanent standards with a rather simple comparator block,* shows that the accuracy of the method is great enough to advance it into the rank of adequate quantitative methods. In Table 5 are shown the results obtained by it as compared with determinations run by the copper-iodometric reagent of Shaffer and Somogyi.²

This method is as rapid and simple as a properly executed qualitative test for urinary sugar, particularly when one is to carry out considerable numbers of determinations. When only occasional single determinations are to be made, however, preliminary heating of a water bath to boiling and heating of the reaction mixture for eight minutes may involve a waste of time. This difficulty can be overcome by the use of an open flame. Analysis is carried out as follows:

Technic.—

Measure into a test tube 0.5 c.c. of urine and 5 c.c. of the carbonate reagent, drop in two small glass beads or quartz pebbles, and a drop of paraffin oil (or a corresponding bit of paraffin wax), heat to boiling over an open flame and, turning the flame low, keep the liquid boiling *very gently* for one and one-half to two minutes. The color develops to the same shade as after heating for eight minutes in a boiling water bath and can be matched against the permanent standards.

A distinct advantage of the carbonate reaction is that it is more specific for sugars than are copper reagents. This quality comes mainly into play when dealing with concentrated urines, so frequent in hot summer weather, which may reduce appreciable amounts of copper without containing any abnormal amounts of glucose; with Somogyi's method such urines give negative results.

Somogyi further states that if the urine is abnormally dark colored or contains blood or bile, it must be decolorized preliminary to the analysis. To this end add about 2 to 3 gm. (roughly one-half teaspoonful) of Lloyd's reagent†

*Sets of permanent standards with a matching block were placed on the market by A. S. Aloe Co., St. Louis, Mo., and are available through laboratory supply houses.

†A hydrated aluminum silicate used extensively in urine analysis for clarification; also for adsorption of certain alkaloids. Obtainable from Hartman-Leddon Co., Philadelphia.

¹Somogyi: Loc. cit.

²Shaffer, P. A., and Somogyi, M.: J. Biol. Chem. 100: 695, 1933.

to about 10 c.c. of urine in a test tube or a small flask, agitate the mixture gently for about a minute (or longer), and then filter through a dry filter paper. The nearly colorless filtrate is ready for analysis.

Benedict's Method

Reagents.—

Benedict's Volumetric Solutions.—

(a) Dissolve 18 grams crystalline copper sulphate, c. p., in 100 c.c. distilled water with heat in a small Pyrex beaker.

(b) In a 1,000 c.c. Pyrex beaker, dissolve 100 grams anhydrous sodium carbonate, c. p. (or 200 grams of crystalline sodium carbonate), 200 grams sodium or potassium citrate, c. p., and 125 grams potassium sulphocyanate, c. p., in 600 c.c. distilled water with heat.

(c) Filter. Add the copper sulphate slowly, and with constant stirring, to the above mixture. Rinse the residue of copper sulphate in the beaker into the solution with a few c.c. distilled water. Transfer to a 1,000 c.c., volumetric flask, rinsing the beaker with a few c.c. distilled water, adding the rinse water to the solution in the flask. Add 5 c.c. of a 5% solution of potassium ferrocyanide. Cool and dilute to 1,000 c.c. with distilled water. Mix thoroughly. Twenty-five c.c. of this reagent are reduced by 50 mg. of dextrose, 52 mg. of levulose, 54 mg. of galactose, or 67 mg. of lactose.

To Adjust Benedict's Volumetric Solution.—

1% glucose solution: Dissolve exactly 1 gram glucose in distilled water in a 100 c.c. volumetric flask and dilute to 100 c.c. with distilled water. This solution is not stable.

Place in a casserole 25 c.c. of newly made Benedict's volumetric solution.

Add a few grams of sodium carbonate and a pinch of powdered pumice.

Titrate as follows:

Fill a buret with 1% glucose. Record the reading on the buret. Boil the contents of the casserole.

Add 1% glucose drop by drop to the casserole, boiling constantly, until the copper sulphate is reduced to a gray color. Record the reading on the buret. Subtract the first reading from the second.

If more than 5 c.c. of a 1% solution of glucose is used, the Benedict's volumetric solution is too strong. Adjust as follows:

Example.—

6 c.c. of a 1% glucose solution were used.

6 c.c. contain 60 mg. of glucose.

$$60 : 50 :: x : 25$$

$$50x = 1500$$

$$x = 30 \text{ (volume to which each 25 c.c. of solution must be diluted).}$$

30 - 25 = 5 c.c. of water must be added to each 25 c.c. of Benedict's volumetric solution. There are 975 c.c. of Benedict's volumetric solution on hand.

$$975 \div 25 = 39$$

$$39 \times 5 = 195 \text{ c.c. of distilled water to be added to a volume of 975 c.c.}$$

If the solution is too weak:

Example.—

Second reading 23.0

First reading 18.3

4.7

4.7 c.c. of 1% glucose were used to reduce 25 c.c. Benedict's volumetric solution.

4.7 c.c. of 1% glucose contain 47 mg. glucose.

$$47 : 50 :: x : 450$$

$$50x = 21,150$$

$$x = 423 \text{ mg. copper sulphate present in each 25 c.c. of Benedict's}$$

volumetric solution. Each 25 c.c. should contain 450 mg. copper sulphate. This solution lacks 27 mg. copper sulphate for each 25 c.c. Benedict's volumetric solution.

Volume 975 c.c.

$$975 \div 25 = 39$$

$39 \times 27 \text{ mg.} = 1,053 \text{ mg. or } 1.053 \text{ gm.}$ copper sulphate to be added to 975 c.c. Benedict's volumetric solution.

Technic of Benedict's Quantitative Estimation of Glucose in Urine.—

Place the urine in a graduated buret.

Place in a casserole of at least 200 c.c. capacity:

25 c.c. Benedict's volumetric solution (page 54)

5 to 10 grams sodium carbonate

A pinch of powdered pumice

Heat the mixture in the casserole to boiling.

Add the urine from the buret, drop by drop, and very slowly, stirring constantly, boiling the mixture in the casserole throughout the entire procedure. Continue, adding the urine slowly until the last trace of the blue color disappears. This is the end point.

Read from the buret the number of c.c. of urine used in the reaction. Calculate the quantity of sugar present as follows: 25 c.c. of Benedict's volumetric solution are reduced by 50 mg. of glucose. The number of c.c. of urine used in the titration, therefore, contains 50 mg. of glucose. Divide 50 by the number of c.c. of urine used in the titration, to obtain the number of mg. in 1 c.c. of urine. Multiply by 100 to obtain the number of milligrams of glucose in 100 c.c. of urine. Divide this figure by 1,000 to obtain the number of grams of glucose in 100 c.c. of urine, or the per cent of sugar present in the specimen.

Example.—

2.5 c.c. of urine were used in the titration of the specimen.

50 divided by 2.5 is 20.

1 c.c. of urine contains, therefore, 20 mg. of glucose.

100 c.c. contain $100 \times 20 \text{ mg.}$ or 2,000 mg. of sugar.

2,000 mg. are 2 gm. The percentage of sugar present is therefore 2.

Fehling's Method

Ten c.c. Fehling's solution are reduced by 0.05 gram of sugar. Dilute 1 c.c. Fehling's solution with 4 c.c. distilled water in a test tube, and heat to boiling.

Add urine from a 1 c.c. graduated pipette, a drop at a time until all the blue color has been removed from Fehling's solution. The number of c.c. of urine used in bringing about this reaction contained 0.005 gram glucose.

If 0.1 c.c. of urine were used in the reduction of Fehling's solution, then 0.1 c.c. urine contains 0.005 gram sugar.

1 c.c. contains 0.05 gm. sugar.

100 c.c. urine contains 5 grams. The percentage of sugar in the urine is, therefore, 5.

Robert's Fermentation Test*

Place 4 ounces of urine in each of two bottles; the first, a bottle of 12 ounces capacity; the second, of 4 ounces capacity. Add a piece of fresh yeast the size of a walnut to the urine in the 12 ounce bottle. Cork. Nick the cork for the escape of gas evolved by the fermentation of the sugar. Cork the smaller bottle tightly. Keep both bottles at room temperature. The fermentation of the sugar will be completed within twenty-four hours. Take the specific gravity of urine in both bottles. The difference of the specific gravities indicates the number of grains of sugar per fluid ounce.

*Use brewer's or compressed yeast. This is positive for dextrose (glucose), levulose (fructose), and galactose.

Example.—If the specific gravity of the unfermented urine is 1.035 and of the fermented urine is 1.020, the urine contains 15 grains of sugar per fluid ounce, or 3%.

This method is not absolutely accurate.

Einhorn's Fermentation Saccharometer*

Use an Einhorn saccharometer. It is a fermentation tube on a stand with graduations on the arm. It is necessary to have two such tubes and one test tube for a test.

Place 10 c.c. urine in a test tube, and add 1 gram of fresh yeast. Shake thoroughly. Pour into the saccharometer, tilting it back until the fluid runs up into the arm, expelling all the air.

At the same time, fill a saccharometer with normal urine.

Allow both to stand at room temperature for twenty-four hours.

The tube containing the normal urine should show no gas. A small bubble of gas may be disregarded. If the normal urine reacts correctly, the test may be read. Read from the arm of the fermentation tube the amount of sugar present. If the urine contains more than 1 per cent of sugar, it must be diluted with distilled water before use.

Urine of a specific gravity of 1.018 to 1.020 is diluted twice. Urine of a specific gravity of 1.021 to 1.028 is diluted five times. Urine of a specific gravity of 1.029 to 1.038 is diluted 10 times.

Millard-Smith Micro-Modification

Place exactly 1 c.c. Benedict's volumetric reagent in a test tube.

Add 0.2 to 0.7 gram anhydrous sodium carbonate, and a pinch of powdered pumice.

Draw the urine into a Mohr pipette up to the 100 mark.

Heat the Benedict solution to boiling.

Add the urine from the Mohr pipette until reduction of the copper sulphate is complete, determined by the disappearance of the blue color. Urines containing more than 3% of sugar should be diluted before titration. Calculate the quantity of sugar present.

1 c.c. Benedict's volumetric reagent is reduced by 2 mg. glucose. If 2 c.c. urine were used, then 2 c.c. urine contained 2 mg. glucose. 1 c.c. urine contained 1 mg. glucose. 100 c.c. urine contained 100 mg., or 0.1 gram glucose, or 0.1%.

Levulose in Urine

Seliwanoff's Reaction

To 10 c.c. urine in a test tube, add a small amount of resorcin and 2 c.c. diluted hydrochloric acid, and mix. Heat.

If levulose is present, the solution turns red and precipitates a dark sediment. The sediment is soluble in alcohol, giving a bright red color.

Lactose in Urine

Rubner's Test

To 10 c.c. urine in a test tube, add 3 grams lead acetate. Filter off the precipitate, and heat the filtrate for a few minutes. A yellowish brown color appears. At this point add ammonium hydroxide and continue heating.

If lactose is present, a brick red color appears in the solution, with a cherry red or copper-colored precipitate. The supernatant fluid becomes colorless after the formation of the precipitate.

Lactose may be found in the urine of women during lactation and in patients who have been on an exclusive milk diet for a long time.

Maltose or Isomaltose in Urine

Maltose or isomaltose reduces copper solutions, but not so strongly as glucose does. Ten c.c. of Fehling's solution are reduced by 0.0807 gram maltose.

*Use brewer's or compressed yeast. This is positive for dextrose (glucose), levulose (fructose), and galactose.

Cane Sugar, Saccharose, in Urine

Pure saccharose does not reduce Fehling's solution.

The finding of saccharose in the urine is of no clinical importance.

Pentoses in Urine

The more important pentoses found in urine are rhamnose, arabinose, and xylose. The pentoses reduce copper solutions, the reduction taking place more slowly than with other carbohydrates.

The presence of pentose may be only a temporary or alimentary disturbance due to the ingestion of large amounts of pentose-rich fruits, but it may be pathologic, especially in diabetes. The pentose which is most frequently present in the chronic form is arabinose.

Ten c.c. Fehling's solution are reduced by 0.0542 gram pentose.

Fermentation test is negative.

Nylander's solution yields a gray precipitate.

Bial's Orcin Test for Pentoses

Bial's reagent.—

30% Hydrochloric acid	500 c.c.
Orcin	1 gram
10% Ferric chloride solution	25 drops

Technic.—

Boil 5 c.c. Bial's reagent in a test tube.

Remove from the flame, and add urine, drop by drop, until not more than 1 c.c. urine has been used. A green color appears almost immediately if pentoses are present.

ALBUMIN

Significance of Albuminuria

Tests for albumin in urine depend upon the visual demonstration of the precipitated protein. The question of what is or what is not pathologic has not entirely been settled. The problem as to which is the best test for protein or albumin is also the subject of much debate. The sulphosalicylic acid test is sixteen times more sensitive than Heller's test when applied to dilute protein solutions, and when applied to urine the sensitiveness is three times as much.

Folin* evidently realized the danger of introducing too sensitive a test for routine work and advised a method of using sulphosalicylic acid which is ten to fifteen times less sensitive than the maximum which can be obtained. Knowledge of the significance of minute amounts of albumin will be enhanced by the development of quantitative methods using sulphosalicylic acid.

It is generally conceded that the tests detailed in this book represent those commonly used. Whether the albumin is present in amounts of a faint trace, a trace, a small amount, a moderate amount, or a large amount, we believe the method suggested by Kingsbury and Clark the most accurate and logical procedure.

A much more important question is the significance of small amounts of albumin in urine. According to life insurance companies, albuminuria in amounts up to 0.03 per cent does not prevent a person under thirty-five years

*See Berglund, Medes, et. al.: The Kidney in Health and Disease, Philadelphia, 1935, Lea & Febiger, p. 441.

of age, and with a negative previous history and otherwise normal findings, from being accepted as a standard risk. When individuals, however, with a minute trace of albumin give a history of previous renal disease, then, of course, this finding becomes of great significance.

Insurance authorities, according to Daly,¹ divide albumin groups dependent upon the amount of albumin found in various specimens. Daly further divides them into two other classes: *Intermittent*, in which the albumin is found in two-thirds or less of the specimens examined, and *constant*, in which albumin is found more often than above. An estimate of the Medico-Actuarial Committee, published in 1938, states that in the class of the *intermittent small amounts*, there were 51,807 exposed to risk, 298 actual deaths against the expected death total of 232, or a mortality ratio of 128 per cent of the expected; in the class of *intermittent moderate amount*, 5,881 with 23 actual deaths against an expected death total of 17 deaths, or a mortality ratio of 132 per cent. According to figures of insurance authorities, the mortality is fairly constant in the *intermittent* group, but this does not hold when the albumin is *constant*. In the *moderate amount* or *constant* albuminuria group, there were 86 actual deaths against 52 expected, with a mortality ratio of 165 per cent, and in the *large amount* category, there were 50 actual deaths against an expected 17, with a mortality ratio of 289 per cent. Expressed in other words, the *constancy* of albuminuria has a definite primary influence on mortality, the *amounts* only a secondary influence. However, the mortality ratio was higher at the older than at the younger ages of entry and increased as the duration of the policies advanced, according to Daly's figures. He states further that the concept of renal disease has changed. Formerly, albuminuria and hypertension were considered definite evidence of Bright's disease; now it is recognized that the hypertension usually comes first and the prime effect is on the cardiovascular system and only secondarily on the kidney. This is reflected in the mortality statistics of insured lives.

The presence of albumin in the urine does not necessarily mean any disease of the kidney, for it may at times reflect the reaction to substances, toxic and otherwise, that originate within the body. Physiologic factors may cause transient albuminuria, such as orthostatic and lordotic albuminuria, which will be explained later. In the insurance world, albuminuria is but a symptom; albuminuria is considered only from a statistical standpoint. Statistically, one can say that the constancy of albuminuria has a definite primary influence on mortality, the amounts only a secondary influence. However, as stated above, the mortality ratio, according to Daly, was higher at the older than at the younger ages of entry and increased as the duration of the policies advanced. The death rates from nephritis and heart impairment were five and two and one-half times the normal, respectively.

Albuminuria may be classified clinically as follows:

1. **Febrile albuminuria** which usually disappears with the fever.
2. **Toxic albuminuria** which may be either chemical or bacterial: chemical poisoning by phenol, phosphorus, lead, mercury, arsenic, etc.; bacterial

¹Daly, Robert M.: *Urol. & Cutan. Rev.* Jan., 1942.

toxic albuminuria such as is seen in bacterial diseases: diphtheria, scarlet fever, meningitis, typhoid fever, etc.

3. **Albuminuria due to cardiovascular disease.**
4. **Hemic albuminuria**; for example, following leukemia, purpura, syphilis, diabetes, etc.
5. **Obstructive albuminuria** caused by ureteral obstruction from stricture, calculus, or pressure of tumors.
6. **Nephritic albuminuria.** This will be fully discussed later. (See pages 174 and 181.)
7. **Neurotic albuminuria** such as is seen in certain diseases of the central nervous system; such as epilepsy, brain tumor, cortical injuries, etc.
8. **Alimentary albuminuria.** This is an albuminuria that occurs in certain individuals from undue ingestion of protein material.
9. **Accidental albuminuria** where the albumin comes from other material containing albumin mixed with the urine; such as, pus, blood, seminal fluid, etc.
10. **Physiologic albuminuria.** This occurs from violent physical exercise. The author some years ago made an extensive study of the urine of marathon runners and found albumin in the urine of all men who participated in the 25 mile race. This was a transitory condition.
11. **Postural albuminuria.** This is described below.
12. **Benign albuminuria** occurs in childhood and adolescence, occurring also in cycles, hence frequently called "cyclic" albuminuria.

Albuminuria is frequently found in young men without any other indications of pathology. Statistical studies in young men with albuminuria disclosed very little difference eventually between the normal subjects and those with albuminuria. In other words, transitory albumin is likely to be found in young men. College students have been examined, for instance, and have been found to have definite albuminuria in a certain percentage of certain groups. Examination from five to ten years later revealed that in most cases the albuminuria had disappeared.

The past history of these young people must be taken into account in estimating the significance of an albuminuria. Those with frequent colds or disturbances in the nose and throat were frequently shown to have albuminuria.

Many different names have been given to this transitory albuminuria of supposedly healthy people, such as "orthostatic," "postural," "lordotic," "juvenile," "pubertile," "cyclic," "transitory," etc.

In relation to the so-called postural type of albuminuria, albumin frequently appears in the urine of young men only when they are in an erect posture. Jehle¹ noted that most of these men with postural or orthostatic albuminuria showed in the standing position a well-marked lordosis, the deepest part of which was at the level of the first and second lumbar vertebrae. This disappeared when the patient reclined. Jehle proved that the production of a lordosis in the erect position accounted for the orthostatic nature of the albuminuria. If a person remained on his knees, a position in which lordosis is very marked, the albuminuria was very pronounced. If the patient, while stand-

¹Jehle: *München. med. Wchnschr.* 55: 12, 1908.

ing, put one foot on a stool, thus eliminating the lordosis, the albuminuria disappeared. It seems undeniable that the cause of albuminuria in many young people is a lumbar lordosis.

The albuminuria which most concerns us as an important diagnostic factor is that seen usually in nephrosis or nephritis. In diseases of the kidney there is an alteration in the secretory function to such an extent that leakage of protein occurs. The urinary protein in renal disease consists of serum albumin and globulin, which are eliminated from the blood plasma. Occasionally in renal disease a cloud forms after the addition of a few drops of 5 per cent acetic acid to the cold urine. Proteins giving this reaction are known as the acetic acid body. This body does not coagulate on heating. According to Moerner² and Pollitzer,³ it is the result of the presence in the urine of serum albumin and chondroitin-sulphuric acid, which precipitates protein at a faintly acid reaction. In most forms of renal disease, albumin is far more abundant than globulin in the urine.

The amount of protein in albuminous urine varies greatly. The most copious albuminuria occurs in degenerative processes in the renal epithelium; especially great quantities of protein are found in the urine in syphilitic and other chronic nephroses. As much as 5 per cent protein may be found in syphilitic nephrosis.

Regarding the transit of albumin from blood to urine, it seems that urinary proteins have different isoelectric points from the corresponding serum proteins; therefore these must undergo some modification in their transit through the kidney or in the urine.

Some have claimed that proteins are derived entirely from disintegration of the renal cells, but this is questionable. Everything points to the fact that the amount of protein derived from the tubular epithelium is evidently small in comparison with that coming from the blood.

Most authorities contend that the albumin filters through the glomerular portion of the kidney rather than through the tubular portion. A most interesting experimental observation was published by Bieter.⁴ By means of asphyxia, the intravenous injection of hemoglobin, the administration of mercuric chloride, and other procedures, he produced albuminuria in fish with kidneys containing glomeruli. On the other hand, these same experimental procedures failed to produce albuminuria in aglomerular fish, that is, fish with kidneys composed entirely of tubules and containing no glomeruli. His experiments served to prove that protein can pass from the plasma into the urine only through the glomeruli. We must accept the idea, therefore, that the major part of the albumin filters through the glomerular portion of the kidney in cases of renal disease, particularly chronic nephrosis.

The manner in which protein is excreted in the glomerular portion has likewise been the subject of much study. The increased permeability of the kidney to albumin is in line with the fact that the smallest molecular parts of plasma proteins alone pass into the urine. The serum albumins are of smaller molecular

²Moerner: *Skandinav. Arch. f. Physiol.* 6: 332, 1895.

³Pollitzer: *Deutsche med. Wchnschr.* 38: 503, 1912.

⁴Bieter: *J. Pharmacol. & Exper. Therap.* 43: 407, 1931.

weight than the serum globulins, and albuminous urines almost always contain a much higher ratio of albumin to globulin than does the blood. We do not know, of course, what particular influence produces the increased permeability in the glomerular portion.

(a) Qualitative Tests

Heat Test with Acetic Acid

Fill an ordinary test tube about $\frac{1}{4}$ or $\frac{1}{6}$ full of urine.

Boil thoroughly. Add 2 or 3 drops of a mixture of equal parts of glacial acetic acid and water.

If albumin is present, the urine becomes cloudy; the greater the cloudiness, the larger the amount of albumin.

The following reactions are possible:

1. If the urine is transparent, and remains so, it is normal.
2. If the urine becomes cloudy after boiling, but clears after the addition of acetic acid, phosphates are present. If the urine effervesces upon the addition of the acid, either calcium carbonate or ammonium carbonate is present.
3. If the urine becomes cloudy after boiling, and remains cloudy or increases in cloudiness, albumin is present. If albumin is present in large quantities, flakes will form and precipitate. Slight cloudiness may be detected by comparison of the test with a test tube of the untested urine. If the two test tubes are held against a black background, the faintest cloudiness may be observed.
4. If the urine is cloudy, and clears upon boiling, and remains clear after the addition of the acid, urates are present, especially sodium urate.
5. If the urine is cloudy, and the cloudiness disappears upon warming, but reappears and becomes more pronounced upon boiling with the addition of the acid, urates are present in excess.
6. If the urine is cloudy, and remains unchanged by boiling and the addition of the acetic acid, microorganisms are present.

Purdy's Modification of the Heat Test with Acetic Acid

This test is made to eliminate the possibility of nucleo-albumin and mucin.

Filter the urine. Fill a test tube about $\frac{2}{3}$ full of the filtered urine. Add $\frac{1}{6}$ its volume of saturated aqueous solution of sodium chloride.

Add 5 to 10 drops of 50% acetic acid. Gently heat the upper inch or so of the mixture for half a minute. If albumin is present, it appears as a cloud in the upper, boiled portion of the test tube. It must be examined in a good light. Even the minutest traces of albumin may be detected by this test.

Heller's Test

Place a small quantity of pure nitric acid in a test tube.

Stratify a small amount of urine on the nitric acid.

A white ring at the point of contact indicates the presence of albumin. Urates appear in a ring above the point of contact. Uric acid and urea are recognized by their crystalline nature. Mucin is precipitated, but is dissolved by the nitric acid at the point of contact. Nucleo-albumin and albumoses are precipitated. Nucleo-albumin gives a fainter ring than albumin, and is above the point of contact. Albuminoses disappear upon heating, and reappear upon cooling. They are also soluble in picric acid.

Potassium Ferrocyanide Test

Place 10 c.c. of filtered urine in a test tube and add 5 drops of glacial acetic acid. A precipitate should be removed by filtration.

Add a few drops of a 5 to 10% solution of potassium ferrocyanide. If a small amount of albumin is present, a faint cloudiness at once appears. If a large amount of albumin is present, a flocculence occurs. Albumose gives a cloudiness, which disappears upon heating.

Spiegler's Test (Modified by Jolles)

Reagent.—

Mercuric chloride -----	10 gm.
Succinic acid -----	20 gm.
Sodium chloride -----	20 gm.
Distilled water -----	500 c.c.

Technic.—

Filter the urine. Place 5 c.c. of filtered urine in a test tube and add 1 c.c. of a 30% acetic acid solution. Stratify with 4 c.c. of Spiegler's reagent.

If albumin is present, a white zone will be formed at the point of contact.

Sulphosalicylic Acid Test

Place 4 or 5 c.c. of urine in a test tube and add 2 or 3 drops of a 20% aqueous solution of sulphosalicylic acid, and mix thoroughly.

Opalescence occurs if small amounts of albumin are present. If large quantities of albumin are present, a pronounced turbidity is noted. A heavy precipitate may accompany the turbidity. Albumose is precipitated, but disappears on heating, reappearing on cooling.

Biuret Reaction

Place the urine in a test tube, and treat first with 10% sodium or potassium hydroxide solution, then with a 10% cupric sulphate solution. The copper sulphate is added drop by drop.

If serum-albumin and globulin alone are present, the mixture becomes pure violet. If albuminoses and peptone alone are present, it turns rose. If several of the albumins are present together, the urine assumes tints between violet and rose.

Do not add too much copper sulphate, because its color will obscure a faint reaction.

Picric Acid Test

Filter the urine. Place 5 c.c. urine in a test tube. Add an equal amount of saturated solution of picric acid.

If albumin is present, a precipitate forms, varying from a light cloud to a heavy flocculence, according to the quantity of albumin.

Albumoses are dissolved.

Robert's Test

Reagent.—

Saturated aqueous solution of magnesium sulphate (made by adding 76.9 gm. magnesium sulphate to 100 c.c. distilled water)-----	5 parts
Concentrated nitric acid, c. p. -----	1 part

Technic.—

If the urine is cloudy, filter.

Place 1 c.c. Robert's reagent in a test tube.

Stratify 1 c.c. of urine on the reagent.

A white ring at the point of contact indicates albumin; the larger the ring, the greater the quantity of albumin. Albuminose also precipitates with this reagent, but is soluble in picric acid. We highly recommend this test.

(b) Quantitative Tests for Albumin in Urine

Kingsbury-Clark's Method

The advantages of this test are:

1. Uric acid and resin do not react with sulphosalicylic acid.
2. The mucin-like substances are not appreciably affected.
3. The definite amount of albumin is ascertained.

Pipette 2.5 c.c. of centrifuged urine into a test tube graduated at 10 c.c. and add 3 per cent sulphosalicylic acid (30 grams dissolved and diluted to 1,000 c.c. with distilled water) to the 10 c.c. mark. Invert the tube to mix, allow to stand 10 minutes, and compare the turbidity with the permanent turbidity standards. Record the value of the standard most closely matched, as the albumin content of the urine. Seven standards are used, equivalent to 10, 20, 30, 40, 50, 75, and 100 milligrams of albumin per 100 c.c. of urine.* In case the specimen contains more than 100 mg. albumin it may be quantitatively diluted and re-determined. By multiplying the value found by the number of times the specimen has been diluted, the desired result is obtained.

A detailed description of the method will be found in the original paper by Kingsbury, Clark, Williams, and Post.¹

A value of 50 mg. of albumin per 100 c.c. of urine obtained by this method corresponds to what is usually designated as a "trace" when Heller's or the heat and acetic acid test is used.

Although the albumin standards are referred to as being permanent, this is not strictly true. After about nine months the lighter standards tend to become more so and thus yield results which are somewhat too high. The entire set of standards should be replaced every nine months.



Fig. 16.—The Kingsbury-Clark albumin kit. (Courtesy Fales Chemical Co., Cornwall Landing, N. Y.)

The principle of the method is that clarified urine is treated with sulphosalicylic acid and the degree of turbidity is measured by comparison with standard turbidities.

The seven standards, representing 10, 20, 30, 40, 50, 75, and 100 mg. albumin per 100 c.c. of urine, are corresponding degrees of turbidity uniformly suspended in gelatin medium. Carefully matched test tubes are used for containing the standard turbidity masses to compare perfectly with the empty calibrated specimen test tube in which the test is conducted. See page 148 for method of making these standards.

The urine should be filtered or centrifuged. Comparison of the test with the various standards is made with a special comparison rack, and the albumin content is recorded as the value of the standard it most closely matches in turbidity. The comparison rack is best located so that uniform north light enters through the rear, striking through the standards and viewed by the observer at an eye elevation of about 12 inches or so.

It goes without saying that the calibrated tubes in which the test is carried out should be of the same dimensions as the standards.† The standards, when not in use, should be kept in the daylight rather than in a dark cupboard to prevent taking on a dark green tinge. Exposure to undue heat will prove harmful to the standards.

*See p. 148.

†These tubes, with the standards, can be obtained from the Fales Chemical Company, Cornwall Landing, New York.

¹Kingsbury, Clark, Williams, and Post: *J. Lab. & Clin. Med.* 11: 981, 1926.

See also Hawk, Oser, and Summerson: *Practical Physiological Chemistry*, ed. 13, Philadelphia, 1954, The Blakiston Co., p. 929.

Esbach's Method

Esbach's Reagent.—

Dissolve 1 gram picric acid, c.p.
And 2 grams citric acid, c.p., in distilled water.
Dilute to 100 c.c. with distilled water.

Technic.—

Render the urine acid by adding a few drops of diluted hydrochloric acid, testing with blue litmus paper after the addition of each small quantity; then filter. If the quantity of albumin is very great, dilute accurately with distilled water before making the test.

Fill the Esbach tube to the mark "U" with urine.

Add Esbach's reagent until the liquid stands at the mark "R." Take care to prevent the formation of bubbles. Close with a rubber stopper.

Mix by inverting several times, and allow to stand in an upright position for 24 hours. Read from the tube the height of the coagulum, which will be the number of grams of albumin in 1,000 c.c. urine. To obtain the percentage, divide this by 10. If the urine has been diluted, multiply the final result by the dilution.

Purdy's Method

Place 10 c.c. urine in a graduated 15 c.c. centrifuge tube.

Add 2 c.c. 50% acetic acid.

Add 3 c.c. 10% potassium ferrocyanide.

Mix, and allow to stand for ten minutes.

Centrifuge at 1,500 r.p.m. for three minutes.

Read off the volume of precipitate on the test tube.

Use Table 6 for calculations. The equivalent on the table gives the number of grams of dry albumin in 100 c.c. urine. If the volume of the 24-hour specimen is 1,500 c.c. and the precipitate in the centrifuge tube stood at 1.2, then the number of grams of dry albumin in the 24-hour specimen is as follows:

Equivalent to 1.2 in the table is 0.25 gram per 100 c.c. In 1,500 c.c. of urine, there are 15 times 0.25 gram, or 3.75 grams. If the amount of albumin is very large, dilute the urine accurately, and after calculation, multiply the final result by the dilution.

TABLE 6. SHOWING THE RELATION BETWEEN THE VOLUME OF PRECIPITATE AFTER CENTRIFUGATION AND THE GRAVIMETRIC PERCENTAGE OF ALBUMIN

VOLUME OF PRECIPITATE C.C.	PERCENTAGE BY GRAMS OF DRY ALBUMIN	VOLUME OF PRECIPITATE C.C.	PERCENTAGE BY GRAMS OF DRY ALBUMIN	VOLUME OF PRECIPITATE C.C.	PERCENTAGE BY GRAMS OF DRY ALBUMIN
0.05	0.01	1.4	0.292	3.2	0.667
0.10	0.021	1.5	0.313	3.3	0.687
0.15	0.031	1.6	0.333	3.4	0.708
0.20	0.042	1.7	0.354	3.5	0.729
0.25	0.052	1.8	0.375	3.6	0.75
0.30	0.063	1.9	0.396	3.7	0.771
0.35	0.073	2.0	0.417	3.8	0.792
0.40	0.083	2.1	0.438	3.9	0.813
0.45	0.094	2.2	0.458	4.0	0.833
0.50	0.104	2.3	0.479	4.1	0.854
0.60	0.125	2.4	0.5	4.2	0.875
0.70	0.146	2.5	0.521	4.3	0.896
0.80	0.167	2.6	0.542	4.4	0.917
0.90	0.187	2.7	0.563	4.5	0.938
1.0	0.208	2.8	0.583	4.6	0.958
1.1	0.229	2.9	0.604	4.7	0.979
1.2	0.25	3.0	0.625	4.8	1.0
1.3	0.271	3.1	0.646		

Kwilecki's Modification of Esbach's Method

Place the urine in the Esbach tube to the mark "U." Add 10 drops of 10% solution of ferric chloride and mix. Fill the tube to the mark "R" with Esbach's reagent, and mix. Place in a water-bath at 72° C. for a few minutes. The result may be read at once.

Tsuchiya's Modification of Esbach's Method**Tsuchiya's Reagent.—**

Crystalline phosphotungstic acid	1.5 gm.
Concentrated hydrochloric acid	5 c.c.
95% alcohol	93.5 c.c.

Use this reagent instead of the Esbach reagent in the Esbach tube, and read after twenty-four hours.

LaMotte Outfit for Determination of Albumin and Sugar in Urine

The LaMotte albumin and sugar outfit is very useful for simple, accurate, quantitative tests for albumin and sugar in urine. The Kingsbury-Clark sulphosalicylic acid method is used for the albumin determination, and the Benedict picrate method for sugar.

Urine is treated with sulphosalicylic acid solution, and the resulting turbidity is compared with prepared turbidity standards. The percentage of albumin in the urine is obtained from the value on the label of the tube with which a match is obtained.

The sugar determination involves treating the urine with picric acid in alkaline solution, and development of the color by heating in a water bath. Per cent of sugar is obtained by comparison with permanent color standards.

PROTEOSES IN URINE

Both deuteroproteose and heteroproteose are found in urine in pathologic conditions. They may be divided into two groups; namely, *primary* and *secondary*. Primary proteoses are precipitated upon half saturation with ammonium sulphate and secondary proteoses upon complete saturation.

Acidify the urine with acetic acid and filter off any precipitate of nucleoprotein which may form.

Boil for several minutes.

Filter while hot to remove the albumin and globulin.

Test the filtrate by overlaying a saturated solution of trichloroacetic acid. A white ring at the point of contact indicates the presence of proteoses. If the test is positive, the primary and secondary proteoses may be separated by half and complete saturation with ammonium sulphate.

Identification of Bence-Jones Protein

Significance of Bence-Jones Protein.—The presence of Bence-Jones protein in the urine of a patient suspected of having multiple myeloma is supportive evidence of this disease, but it is not diagnostic. This protein is present in almost all cases of multiple myeloma during any active phase of the disease. The protein may not be found in the urine when the disease is not actively spreading. Any disease which is associated with rapid destruction of bone and displacement of bone marrow may be associated with a positive Bence-Jones test.

Cope, in a round table discussion of this question,¹ reported that it was present in two of his cases of hyperparathyroidism with classic bone disease,

¹Cope, Oliver: J. Missouri M. A. 30: 273, 1942.

due to a rapid progression of giant cell tumors. It is occasionally present in patients with metastatic malignancy of bone. A positive test therefore suggests multiple myeloma; a negative test does not exclude multiple myeloma.

The first reference in the literature to Bence-Jones protein appeared in *Philosophical Transactions of the Royal Society* for 1848 in a paper presented by H. Bence-Jones on "A New Substance Occurring in the Urine of a Patient With Mollities Ossium." His paper stated, "On the first of November, 1845, I received from Dr. Watson the following note, with a test tube containing a thick, yellow semi-solid substance. The tube contained urine of a very high specific gravity; when boiled it became highly opaque; on the addition of nitric acid it effervesces, assumes a reddish hue, becomes quite clear, but as it cools, assumes the consistence and appearance which you see: heat liquefies it. 'What is it?'"

At the present time, Bence-Jones proteins should be regarded not as chemical entities but rather as a class of proteins whose physical state is altered in vitro by temperature changes. That there are multiple Bence-Jones proteins is further supported by differences in (1) solubility, (2) electrophoretic mobility, (3) sedimentation velocity, and (4) serologic properties of constituent protein substances. Some investigators conjecture that the patient with multiple myeloma excretes this protein, that it is the result of tumor cell metabolites. Ellinger demonstrated these proteins in the blood, also in ascitic fluid. Yet it has been shown that in the presence of a Bence-Jones proteinuria, heat-coagulable serum proteins may be absent.

Martin and Mathieson² suggest the name "pyroglobulinemia" to indicate the presence of heat-coagulable globulin in the blood. This is in contrast to the "cryoglobulins" which are serum protein gels that appear on exposure to cold and redissolve on warming. Cold-coagulable globulins have been described in association with multiple myeloma, and also in association with other conditions. The same applies to heat-coagulable globulins. The cases reported to date have, in the main, concerned victims of multiple myeloma, but Hammarsten and associates referred in their work to sera which are sensitive to heating as being found occasionally in chronic infections and in instances of hyperglobulinemia in otherwise clinically healthy persons. Martin and Mathieson reported one case of pyroglobulinemia associated with multiple myeloma, but in four cases this association did not obtain. In one of the latter cases, the patient had lymphosarcoma. There is at times no correlation between the occurrence of pyroglobulinemia and Bence-Jones proteinuria.

The evidence at hand favors the conclusion that Bence-Jones protein is an extremely small globulin, or group of small globulins, with a molecular weight of approximately 30,000. Its small size may well account for its frequent occurrence in the urine as contrasted with the rarity with which it has been noted in the blood. It has been reported in the following conditions other than multiple myeloma: metastatic carcinoma of the bone, multiple sarcoma of the bone, senile osteomalacia, fibrocystic disease, comminuted fracture, tumor of the jaws, lymphocytic leukemia, myelocytic leukemia, polycythemia, adenocarcinoma of the stomach, carcinoma of the kidney, inactive pulmonary tuberculosis, and prostatitis.

²Proc. Staff Meet., Mayo Clin. 28: 20, 1953.

Qualitative Test.—

Place 10 c.c. of the urine in a test tube in a water bath with a thermometer, and heat very slowly and gently.

Observe frequently. Turbidity will begin to occur at about 40° C. and precipitation will take place at about 60° C.

Now acidulate very slightly with acetic acid and raise the temperature to the boiling point (100° C.). The precipitate now partly or totally disappears. Filter while still boiling hot.

The precipitate returns as the tube cools. Precipitation at so low a temperature and dissolving at a higher temperature is typical of Bence-Jones protein. It is this characteristic which differentiates it qualitatively from all other protein material occurring in urine.

Test the filtrate by overlaying a saturated solution of trichloroacetic acid. A white ring at the point of contact indicates the presence of proteoses. If the test is positive, the primary and secondary proteoses may be separated by half and complete saturation with ammonium sulphate.

(a) Precipitate the protein with nitric acid. This precipitate should disappear on boiling and reappear upon cooling.

(b) Precipitate the protein with alcohol and collect immediately by centrifuging. The precipitate should be soluble in water.

Quantitative Test.—**Reagents.—**

Glacial Acetic Acid.—

50% Ethyl Alcohol.—

Technic.—

Place 10 c.c. urine in a tared centrifuge tube.

Add just enough acetic acid to make it slightly acid.

Place in the paraffin oven overnight in a water bath at 60° C.

Centrifuge and pour off the supernatant liquid.

Add 10 c.c. of 50% ethyl alcohol.

Mix thoroughly and recentrifuge.

Pour off the supernatant liquid and dry the residue at 100° C. to constant weight.

Cool and weigh. Figure the increase in weight.

The increase in weight in 10 c.c. of urine converted to the increase in weight in the entire 24-hour specimen is the amount of Bence-Jones protein present in the sample.

INDICAN

Indican is derived from indol, a product of intestinal putrefaction of albuminous substance. The indol is absorbed by the blood and oxidized in the tissues to indoxyl, which combines with potassium sulphate and is eliminated in the urine. It is present in minute amounts in every normal urine.

Indican is increased following diets rich in protein and in cases of obstruction of the small intestine, also in intestinal indigestion and in diseases of the stomach associated with decreased hydrochloric acid, gastritis, and gastric cancer. It is also increased in diminished bile flow and in cases where there is decomposition of proteins in other parts of the body, as occurs in peritonitis and empyema, and other pus collections.

Determination of Indican in Urine**(a) Qualitative Tests****Obermayer's Method****Obermayer's Reagent.—**

1,000 c.c. fuming hydrochloric acid, c.p.

3 grams ferric chloride, c.p.

1st Method.

Precipitate 20 c.c. urine with 5 to 10 c.c. 10% lead acetate solution and filter.

Add an equal volume of Obermayer's reagent to the filtrate. Shake thoroughly.

Add 5 c.c. chloroform. Shake thoroughly.

A BLUE coloration of the chloroform indicates indican. A PINK color is due to the ingestion of iodides.

2nd Method.

To 5 c.c. urine in a test tube,

add 5 c.c. Obermayer's reagent.

Mix and add 15 drops of chloroform. Shake and allow the chloroform to settle.

A BLUE colorization of the chloroform indicates the presence of indican. A PINK color is caused by ingestion of iodides.

Jaffe's Method

To a small amount of urine in a test tube add an equal volume of hydrochloric acid, c.p.

Mix, and add 10 to 15 drops of chloroform.

Add, drop by drop, a strong fresh solution of calcium hypochlorite, shaking after the addition of each drop. A blue color appears if indican is present.

A 0.5% solution of potassium permanganate may be used in place of the calcium hypochlorite if preferred. Five drops of the potassium permanganate are used with 10 c.c. urine.

Jolle's Method

To 10 c.c. urine add 2 c.c. of 20% lead acetate solution, shake, and filter. To the filtrate, add 0.5 c.c. of a 10% alcoholic solution of thymol

10 c.c. of Obermayer's reagent

4 c.c. chloroform.

Mix thoroughly. The chloroform takes on a pronounced violet color if indican is present.

(b) Quantitative Estimation of Indoxyl Compounds in Urine**Method of Sharlit,¹ modified by Meiklejohn and Cohen²**

Indican, which is indoxyl potassium sulphate, is the most familiar indol derivative in urine. Part of the indol excreted in urine may occur in a form which is responsible for the urochrome reaction (as yet no satisfactory quantitative method has been developed for the latter).

Reagents.—**1% Potassium Persulphate Solution.—**

Dissolve 1 gram of potassium persulphate in enough distilled water to make 100 c.c. of solution.

1% Thymol in 95% Alcohol.—

Dissolve 1 gram of thymol in enough 95% alcohol to make 100 c.c. of solution.

25% Trichloroacetic Acid in Hydrochloric Acid.—

Dissolve 25 grams of trichloroacetic acid in enough hydrochloric acid to make 100 c.c. of solution.

Thymol-free Alcohol.—**Preparation of the Urine for the Test.—**

Place 10 c.c. of unfiltered urine, preserved under toluene, in a 100 c.c. volumetric flask. (It is not necessary to use fresh urine. Addition of a few c.c. of toluene to the urine when first voided is sufficient to maintain the values obtained by this method for at least 24 hours.)

¹Sharlit, H.: J. Biol. Chem. 99: 537, 1932-33.

²Meiklejohn, A. P., and Cohen, F. P.: J. Lab. & Clin. Med. 27: 949, 1942.

Dilute to 100 c.c. with distilled water.

Measure 5 c.c. of diluted urine (containing 0.5 c.c. undiluted urine) into a 15 c.c. graduated centrifuge tube.

Add. 0.5 c.c. of 1% potassium persulphate solution,

0.5 c.c. of 1% thymol in 95% alcohol,

and 5.0 c.c. of 25% trichloroacetic acid in concentrated hydrochloric acid.

Preparation of the Blank for the Test.—

Prepare a blank determination, using urine and reagents in the same quantities as in the test, but substituting *thymol-free alcohol* for the thymol-alcohol reagent.

(High blank readings are sometimes obtained with the use of thymol-free alcohol.

This is due to nonspecific substances which, on oxidation by persulphate, form colored products soluble in ethyl trichloroacetate. Such substances might include urochrome and perhaps bile products. Preliminary treatment with basic lead acetate, as advocated by some workers, may sometimes result in a significant reduction in the colorimeter reading, due to the removal of such interfering pigments.)

Technic.—

Mix the solutions well in their various test tubes.

Place the tubes in a boiling water bath until the ethyl trichloroacetate has filtered out, and the aqueous layer is essentially free from pigment. This requires about 20 minutes.

Centrifuge briefly.

Remove as much as possible of the supernatant aqueous layer, using a pipette.

Take up the trichloroacetate layer, together with the small volume of aqueous layer remaining in the centrifuge tubes, in sufficient glacial acetic acid to bring the volume to the 5 c.c. mark.

Transfer to special tubes for use in a Klett-Summerson photoelectric colorimeter. Thoroughly mix by pouring back and forth between the centrifuge tube and the colorimeter tube.

Read immediately in the photoelectric colorimeter, using a No. 540 filter.

Calculation.—

Subtract the logarithmic scale reading given by the blank determination from the reading of the unknown. Calculate the micrograms of indican per c.c. of urine as follows:

$$\text{Colorimeter reading} \times \frac{0.38}{1000} \times \text{volume of urine} = \text{twenty-four-hour excretion of indican (micrograms).}$$

If the reading on the colorimeter is less than 200 or more than 400 scale units, greater accuracy may be achieved by repeating the test, using appropriately greater or smaller amounts of urine.

Meiklejohn and Cohen³ found that the factor necessary to convert colorimeter readings into terms of the amount of indican present varies somewhat over the range employed; or, in other words, when colorimeter readings are plotted against concentrations of indican, the resulting calibration curve is not absolutely linear, as required to fulfill Beer's law exactly. The differences between the factors for each concentration of indican are so small that for practical purposes one factor may be used throughout. The factor 0.38 is employed to convert colorimeter readings into terms of micrograms of indican per c.c. of solution. This factor was obtained from the following equation:

$$\text{Factor} = \frac{\text{Indican in micrograms}}{\text{Colorimeter reading} \times \text{volume of indican solution}} \text{ or } \frac{50}{262 \times 0.5} = 0.38.$$

³Meiklejohn, A. P., and Cohen, F. P.: J. Lab. & Clin. Med. 27: 949, 1942.

ACETONE BODIES

Diacetic acid, acetone, and β -oxybutyric acid are called the "acetone bodies." Diacetic acid is formed first and the others are derived from it. The chief source of these acetone bodies lies in abnormal catabolism of fats. For further particulars about these bodies, refer to the chapter on Blood Chemistry.

Diacetic Acid

Gerhardt's Method

The urine must be fresh.

To a few c.c. of urine in a test tube add 10% ferric chloride solution drop by drop until no further precipitation occurs.

If diacetic acid is present, the urine will turn a Bordeaux-red color. If the diacetic acid is due to drugs, it does not disappear upon heating. True diacetic acid disappears upon heating, and does not reappear upon cooling. If the reaction is dubious, acidulate some of the urine with sulphuric acid, and shake up with ether. Remove the ether with a pipette, and shake up with very dilute ferric chloride solution. If diacetic acid is present, a violet color results, which changes to a Bordeaux-red upon further addition of ferric chloride.

Arnold's Method, Modified by Liplawski

Reagents.—

Solution No. I

Para-amido-aceto-phenone.....	1 gram
Distilled water.....	100 c.c.
Hydrochloric acid, conc.....	2 c.c.

Solution No. II

Potassium nitrite (KNO_2).....	1 gram
Distilled water.....	100 c.c.

Technic.—

Place 6 c.c. solution No. I in a test tube, and

Add 3 c.c. of solution No. II.

Add an equal volume of urine,

And 1 drop of concentrated ammonia. Shake the mixture until it assumes a brick-red color.

Treat from 10 drops to 2 c.c. of this mixture (according to probable amount of diacetic acid present) with 15 to 20 c.c. of concentrated hydrochloric acid, 3 c.c. chloroform and 2 to 4 drops of an aqueous solution of ferric chloride. Close the tube with a cork, and shake gently for one-half to one minute. If diacetic acid is present, the chloroform becomes tinged with violet. If no diacetic acid is present, the color is yellow or light red.

Acetone in Urine

Legal's Method

Dissolve a few crystals of sodium nitroprusside in 2 c.c. distilled water in a test tube.

To a few c.c. urine, add enough sodium or potassium hydroxide solution to render it distinctly alkaline.

Add a few drops of sodium nitroprusside solution.

Add a few drops of concentrated acetic acid.

A purple or violet-red color indicates acetone. Alcohol or acetic aldehyde and diacetic acid also appear red.

Jackson-Taylor's Modification of Legal's Test

To a few c.c. of urine, add a few drops of a freshly prepared solution of sodium nitroprusside (a few crystals in 2 c.c. water). Mix thoroughly, and add 1 gram am-



PLATE III.—URINE COLOR REACTIONS.

1. Showing Indican Reaction.
2. Showing Benzidine Test for Blood.
3. Showing Acetone Reaction.
4. Showing Diacetic Acid Reaction.

(Gradwohl and Blaivas.)

monium sulphate powder. Mix, and stratify on this 2 c.c. ammonium hydroxide. Allow to stand several minutes.

A purplish-red or permanganate-colored ring at the point of contact indicates the presence of acetone.

Lieben's Iodoform Test

Add a small amount of phosphoric or hydrochloric acid to 200 or 300 c.c. of urine, in the proportion of 3 c.c. to 100 c.c. of urine.

Distill this mixture.

The tests are performed with the first 10 or 15 c.c. of the distillate. To the distillate, add a few drops of strong potassium hydroxide solution and a few drops of iodine and potassium-iodide solution. If acetone is present, a yellow precipitate of iodoform develops at once. This can be easily identified by the characteristic odor. Microscopically, there will be seen thin, yellow hexagonal plates or star-like groups of crystals.

Gunning's Modification of Lieben's Test

To prevent confusion with alcohol, Gunning suggests the addition of an excess of alcoholic iodine solution and some ammonia to the distillate, or to the urine itself. Iodoform is deposited if acetone is present. The liquid often turns black at first, but as the nitrogen iodide settles and disappears, the yellow iodoform deposit can be recognized.

DO NOT WARM THE MIXTURE DURING THE PERIOD THAT IT CONTAINS NITROGEN IODIDE.

Frommer's Test

To 10 c.c. urine in a test tube, add 1 gram of potassium hydroxide. Do not wait for a complete solution.

Treat the mixture with 10 to 20 drops of a 10% alcoholic solution of salicyl aldehyde. Warm to 70° C.

An intense purplish-red ring appears at the point of contact if acetone is present.

Beta-Oxybutyric Acid in Urine

Method of Hart

To 20 c.c. urine add 20 c.c. distilled water and a few drops of acetic acid. Boil, reducing the volume to 10 c.c. This removes the acetone and diacetic acid.

Dilute to 20 c.c. with distilled water.

Mix and divide the contents equally into two portions.

To one of the quantities, add 1 c.c. hydrogen peroxide. Warm gently, and allow to cool.

Apply Legal's test for acetone to both tubes (above). Allow them to stand for a few hours. If beta-oxybutyric acid is present, the tube containing the hydrogen peroxide will show a red ring at the point of contact, while no reaction will be detected in the other tube.

Sodium Bicarbonate Test for Acidosis

Sellard's Method

Every two or three hours the patient is given by mouth 5 grams of sodium bicarbonate dissolved in water, until the urine becomes neutral or faintly alkaline to litmus paper.

Tolerance of 20 to 30 grams of sodium bicarbonate indicates a mild grade of acidosis.

Tolerance of 40 to 50 grams indicates marked acidosis. No clinical symptoms except dyspnea upon excretion are noted. Tolerance of 75 to 100 grams indicates serious acidosis. Clinical symptoms are marked. Tolerance of 150 grams is noted in severe cases. In severe cases of acidosis, do not administer further bicarbonate solution after the existence of marked acidosis has been confirmed.

Detection of Bile in Urine

For a more complete discussion of tests for bile, refer to Chapter VIII, Feces.

These tests depend upon the oxidation of bile pigments by acids with the formation of a series of colored derivatives like biliverdin (green), bilicyanine (blue) and choletelin (yellow). The most important pigment of this group is bilirubin.

Gmelin's Test

Place a small quantity of concentrated nitric acid, containing a small amount of yellow nitrous acid, in a test tube.

Stratify a small amount of urine on the acid.

A band of colored rings at the point of contact of the two fluids indicates the presence of bile. These rings are green, blue, violet, red, and yellow. Green is the most pronounced.

Rosenbach's Test

Filter the urine through pure white filter paper.

Apply a drop of nitric acid containing yellow nitrous acid to the inside of the filter. Around the nitric acid, if bile is present, a band of colored rings will appear.

Ultzmann's Test

To 10 c.c. urine add 3 or 4 c.c. of a 25% potassium hydroxide solution, and an excess of pure hydrochloric acid.

If bile is present, the mixture becomes a definite green color.

Maher Test¹

This qualitative test for bile in the urine was developed during the course of determinations of sulfonamide drugs in urine by the Maher-Camp modification of the Marshall method.²

Place 10 c.c. of urine in a test tube and acidify by the addition of 1 c.c. of a 20 per cent solution of para-toluenesulphonic acid. Ten per cent hydrochloric acid may also be used. Two minutes later, add 1 c.c. of a 0.1 per cent freshly prepared solution of sodium nitrite. Mix the contents of the tube well.

Development of a green color indicates the presence of bile, presumably by the oxidation of bilirubin to a green derivative. No other substances produce a similar reaction.

Determination of the Presence of Bile Acids

Hay's Test

In Hay's test, advantage is taken of the fact that bile acids have the property of reducing the surface tension of fluids in which they are contained.

Cool the urine by placing it in a refrigerator for several hours.

Sprinkle upon the surface a little finely powdered sulphur ("flowers of sulphur").

If the sulphur sinks at once, bile acids are present in the amount of 0.01 per cent or more. If the sulphur sinks only after gentle agitation, bile acids are present in 0.0025 per cent or more. If the sulphur remains floating, even after gentle shaking, bile acids are absent.

Oliver's Test

Oliver's test depends upon the principle that a precipitate is formed of a protein (peptone) and bile acids.

Reagent.—

Peptone	8.33 gm.
Salicylic acid	1.12 gm.
Water containing 2 drops of acetic acid	1,000.0 c.c.

¹Science: 94: 398, 1941.

²Maher, F. T., and Camp, W. J. R.: J. Lab & Clin. Med. 24: 1198, 1939.

Technic.—

Filter a small amount of urine (5 or 10 c.c.) until perfectly clear.

If the urine is neutral or alkaline, acidify with acetic acid.

Dilute with water until the specific gravity is less than 1.008.

Place 2 c.c. in a test tube.

Add 5 c.c. of the reagent.

A positive reaction is indicated by a milky turbidity.

Determination of Urobilin and Urobilinogen

It is claimed that urobilin is excreted as a chromogen, urobilinogen, which is changed by light into urobilin within a few hours after the urine is voided.

Urobilinogen is a normal constituent of urine and, as stated above, is converted into urobilin upon standing. It is increased whenever there is an excess of bilirubin formed through excessive destruction of erythrocytes, especially in pernicious anemia and malaria. When the liver cells fail to function properly, there is an increase, and for this reason the test has become of practical value as a *liver function test*.

A marked decrease or even total absence may occur in obstructive jaundice when the obstruction is complete or nearly complete. If the obstruction is only partial, it may be normal or even increased.

Schlessinger's Test for Urobilin

To 10 c.c. of urine in a test tube add a few drops of Lugol's solution to transform the chromogen into the pigment.

Add 10 c.c. of a saturated alcoholic solution of zinc acetate. Mix and filter.

View the filtrate in sunlight against a dark background or with light concentrated upon it with a lens; a greenish fluorescence indicates the presence of urobilin.

Bile pigment, if present, should be previously removed by adding one-fifth volume of 10 per cent solution of calcium chloride and filtering.

Ehrlich's Test

Reagent.—

Paradimethylaminobenzaldehyde	-----	10 gm.
Hydrochloric acid (conc.)	-----	75 c.c.
Water	-----	75 c.c.

Technic.—**(A) Qualitative test as follows:**

Place 10 c.c. of urine in a test tube 15 millimeters in diameter. The urine should not be too cold. If very cold, allow it to stand at room temperature or gently heat to between 65° and 80° F.

Add 1 c.c. of Ehrlich's reagent and mix.

Allow to stand for from three to five minutes, at the end of which time a cherry-red color appears if urobilin is present in abnormal amount. A light red and shades of pink appear when it is present in normal quantity. The examination for color should be made by viewing the contents through the mouth of the tube, holding it at a slight angle over white paper.

If no color appears, the tube should be allowed to stand longer, and then if no color appears, it should be heated and again examined before reporting an absence of urobilinogen.

This test is to some extent quantitative. With a little experience one can judge by the color whether there is an increase above normal.

(B) A quantitative test may be conducted as follows:

Place six test tubes (15 millimeters in diameter) in a rack. In them place the following amounts of urine:

No. 1: 10 c.c. 1:20

No. 2: 10 c.c. 1:30

- No. 3: 10 c.c. 1:40
 No. 4: 10 c.c. 1:50
 No. 5: 10 c.c. 1:100
 No. 6: 10 c.c. 1:200

The water used for diluting should not be too cold. If tap water is used, it may be necessary to add enough warm water to bring it to about room temperature (between 65° F. and 85° F.).

To each tube add 1 c.c. of Ehrlich's reagent and mix.

At the end of *five minutes* examine the tubes by viewing through the mouth against a white background. Note the highest dilution which shows the slightest pink color. This is reported as positive 1:50, 1:200, etc.

When possible the readings should be made by daylight, as artificial light has a tendency to intensify the color. Highly concentrated urines may give a yellowish brown discoloration which must be differentiated from the true pink reaction of urobilinogen.

Bile pigment, if present, should be previously removed by adding 1 part of 10 per cent solution of calcium chloride to 4 parts of urine, and filtering.

Watson's Quantitative Test for Urobilinogen in Feces and Urine¹

Reagents.—

20% Ferrous Sulphate Solution.—

Dissolve 20 gm. of ferrous sulphate, previously ground to a powder in a mortar, in 92 c.c. distilled water.

10% Sodium Hydroxide Solution.—

Modified Ehrlich's Reagent.—

Dissolve 0.7 gm. of paradimethylaminobenzaldehyde in 150 c.c. of concentrated hydrochloric acid and add to 100 c.c. distilled water.

Saturated Aqueous Solution of Sodium Acetate.—

Petroleum Benzine (Petroleum Ether).—

Glacial Acetic Acid.—

Hellige-Dunning Colorimeter.—

This may be procured from Hynson, Westcott, and Dunning, Inc., Baltimore.

(A) Feces.—

Collect feces for a four-day period, mix well, and grind a 10 gm. portion in a mortar with a few c.c. of distilled water.

Dilute to 300 c.c. with distilled water, or to only 100 c.c. if the feces are definitely acholic.

Pour into an Erlenmeyer flask containing 100 c.c. of 20% ferrous sulphate solution.

Add, with constant mixing, 100 c.c. of 10% sodium hydroxide solution.

Cork and allow to stand one hour. If the flask stands overnight, slightly lower values are obtained.²

Filter.

To 2 or 3 c.c. of the filtrate, add an equal amount of modified Ehrlich's reagent.

Mix and add several c.c. of saturated aqueous solution of sodium acetate.

Depending upon the amount of color, use from 1 to 50 c.c. of the filtrate in the quantitative procedure. If the color is intense, use 1 c.c. or less; if pale red, use 5 to 10 c.c.; if faint, use 25 c.c.; if absent, 50 c.c. The amount of filtrate chosen should be only enough that the final colored solution does not exceed 100 c.c. and that its color is not more than 50 per cent on the standard scale.

Place the amount of filtrate decided upon in a 125 c.c. pear-shaped separatory funnel. Dilute filtrate to 20 c.c. with water if less than this amount of filtrate has been chosen.

Cover with 25 c.c. of purified petroleum benzine.

Strongly acidify with glacial acetic acid.

Shake vigorously for several seconds.

¹Watson, C. J.: Am. J. Clin. Path. 6: 458, 1936.

²Slightly lower values are obtained if the mixture is allowed to stand overnight. For this reason the values given subsequently are undoubtedly slightly too low, since the mixture was allowed to stand overnight in most of these instances.

Separate the aqueous solution of petroleum benzine and wash twice with distilled water. Shake vigorously with 1 or 2 c.c. of modified Ehrlich's reagent.

Add at least twice as much of a saturated solution of sodium acetate. The maximum color now appears.

Shake vigorously, then separate the colored solution into a 100 c.c. graduated cylinder.

Again extract the petroleum benzine with Ehrlich's reagent. Repeat until the urobilinogen has been entirely removed as evidenced by no further development of color.

Follow each extraction by the addition of sodium acetate, shaking, and separation of the aqueous fraction into the cylinder.

Make the colored solution up to a volume convenient for calculation and mix.

Fill the right-hand tube of the Hellige-Dunning colorimeter and compare with the standards. Should the intensity of color be greater than 50 per cent of the standard, dilute the specimen further and read again. Comparisons are most accurate between 20 and 50 per cent.

Calculation.—

$$\frac{\text{c.c. of ferrous hydroxide mixture}}{\text{gm. of feces}} \times \frac{\text{c.c. of final colored solution}}{\text{c.c. of filtrate used}} \times \frac{\text{percentage of color standard} \times \text{weight of 4-day amount of feces}}{100} \times \frac{1}{\text{No. of days' collection of feces}} = \text{mg. of urobilinogen per day.}$$

Example.—

$$\frac{500}{10} \times \frac{45}{3} \times 0.35 \times 2.8 \times \frac{1}{4} = 183.75 \text{ mg. per day.}$$

Standardization of the colorimeter in terms of crystalline mesobilirubinogen (urobilinogen) has been described (Watson, 1936).

(B) Urine.—

Reagents are the same as for feces.

Collect a 24-hour sample of the urine in a dark bottle containing approximately 5 gm. of sodium carbonate and 100 c.c. of purified petroleum benzine.¹

Separate the urine from the petroleum benzine and measure.

Place 50 c.c. of the mixed urine in a 500 c.c. Erlenmeyer flask.

Add, with thorough mixing, 25 c.c. of 20% ferrous sulphate solution and 25 c.c. of 10% sodium hydroxide.

Let stand one hour then make a qualitative determination, then use this information in selecting the amount of urine to be used in the quantitative procedure.

Proceed as with the filtrate from feces. Compare in the Hellige-Dunning² colorimeter.

Calculation.—

$$\frac{100}{50} \times \frac{\text{c.c. of final colored solution}}{\text{c.c. of filtrate used}} \times \text{percentage of standard} \times \frac{\text{vol. of 24 hour urine}}{100} = \text{mg. of urobilinogen in 24 hours.}$$

Example (Hepatic Dysfunction).—

$$2 \times \frac{20}{2} \times 0.18 \times 14 = 50.4 \text{ mg. in 24 hours.}$$

Watson's figures show that the amount of urobilinogen excreted in the urine in twenty-four hours by the normal adult varies between 0 and 4 mg.,

¹In paper I results of comparative estimations following different methods of collecting the urine were given. The majority of the results which are given here and in another paper were obtained after collection of the urine in ferrous hydroxide. It was shown in a previous paper that the collection under petroleum benzene is somewhat more efficient; for this reason the values to be given here are slightly low.

²A permanent glass standard colorimeter for determination of phenolsulphonephthalein in urine made by Hynson, Westcott, & Dunning, Inc., Baltimore.

usually from 0.5 to 2 mg. He noted a great drop in the amount of urinary urobilinogen coincident with the reticulocyte response to liver therapy in cases of pernicious anemia, together with considerable decrease in the amount of urobilinogen in the feces. The normal range for the feces is between 40 and 280 mg. per day, usually from 100 to 250 mg. His conclusions based upon his exhaustive work regarding urobilinogen in feces were that inanition, inactivity, or a low grade infection tends to lower the excretion of urobilinogen; fever of any considerable degree probably tends to increase the amount. He noted increases of the excretion of urobilinogen in feces in cases of pernicious anemia, Hodgkin's disease, and leukemia. Low values were encountered in certain cases of secondary hyperchromic anemia and in all the cases of hypochromic anemia investigated. Slight increases were observed in half the cases of polycythemia vera.

In another paper³ in reference to the excretion of urobilinogen in the common forms of jaundice and diseases of the liver in 135 cases examined, Watson found that jaundice due to stone is not accompanied by a considerable increase in the amount of urobilinogen in the urine unless complications are present, such as acute cholecystitis or cholangitis, biliary cirrhosis incident to persistent biliary obstruction, or severe anemia. These complications are accompanied, according to Watson, by a relatively marked increase, the actual amount of urobilinogen appearing in the urine being dependent to some extent on the degree of biliary obstruction and the consequent amount of urobilinogen formed in the bowel and reabsorbed from it.

Diffuse hepatic disease is characterized by a marked increase of urobilinogen in the urine; some of the instances of idiopathic diseases of the liver in the group of patients with cirrhosis were accompanied by evidence of an increased destruction of blood, i.e., an increase of the urobilinogen content of the feces and regenerative anemia, often macrocytic.

Increases in the amount of urinary urobilinogen in hemolytic jaundice could not be correlated with increased destruction of blood, but it occurred when other factors, which are believed to disturb the function of the liver, also were present. Urobilinogen content of the feces was usually increased to a marked degree, in the majority of cases decreasing rapidly after splenectomy.

Methylene Blue Test for Bilirubin in Urine

Refer to page 331 for the methylene blue test for bilirubin.

Test for Determination of Urorosein

Urorosein does not occur in normal urine but may occur in various diseases, such as pulmonary tuberculosis, typhoid fever, nephritis, and stomach disorders. It is excreted as a chromogen (indolacetic acid) which is transformed into the pigment upon treatment with a mineral acid:

To 10 c.c. of urine in a test tube add 5 c.c. of concentrated hydrochloric acid and a few drops of a 1 per cent solution of potassium nitrite. Mix thoroughly. A rose-red color indicates urorosein.

³Watson, C. J.: *Arch. Int. Med.* 59: 206, 1937.

URINARY PHENOLS

The determination of urinary phenols deserves study because Becher¹ reported the occurrence of an increased amount of these substances in patients suffering with pernicious anemia. Volterra² corroborated this finding. Owing to the difficulties incident to chemical analyses covering these ingredients, attention has been directed to a determination of the urinary phenols. In the determination of isocyclic amino acids, it is also necessary to determine their intermediary products, aromatic hydroxy acids. To this end Volterra³ gives a simple technic for the determination of three fractions of aromatic substances in the urine.

Technic of Volterra.—

Use the Folin and Ciocalteu phenol reagent (see p. 305 for formula for this reagent*). Dilute this reagent with equal volume of distilled water.

To 10 c.c. of fluid in a test tube add 0.5 c.c. dilute phenol reagent, 2 c.c. of 20 per cent sodium carbonate. Shake. After 20 to 30 seconds, immerse the tube in boiling water for exactly one minute and then cool under running water.

This technic is divided into three separate determinations; viz.,

- A. Determination of volatile phenols.
- B. Determination of aromatic hydroxy acids.
- C. Determination of residual phenols.

A. Determination of Volatile Phenols.—

Use 10 c.c. of precisely measured urine from 24-hour specimen. Pour into a 250 c.c. distillation flask. Add 150 to 170 c.c. distilled water and some sodium carbonate until solution is alkaline to litmus paper, then distill. Free volatile phenols are carried over in the steam. They are present in small amounts, hence stop when you have obtained 30 to 40 c.c. After the flask cools, add 2 to 4 c.c. of dilute sulphuric acid until acid to Congo red paper; start distillation again. After 100 c.c. are obtained, place 5 c.c. of the specimen in a small test tube, add 0.25 c.c. phenol reagent and 1 c.c. sodium carbonate; heat in a water bath. If the phenol reaction is still present, continue distillation after adding 100 c.c. of water. To 10 c.c. of both distillates add phenol reagent and sodium carbonate and carry out the test as described. Prepare a standard solution from a stock solution of 1 mg. phenol per c.c. The best suitable standard is that of 3 mg. per cent diluted ten times. This ten-fold dilution has the proper intensity for colorimetric reading.

Calculation.—

$$\frac{\text{Reading standard 15 mm.}}{\text{Reading unknown}} \times \text{mg. in standard} \times \frac{\text{c.c. of distillate}}{100} \times$$

$$\frac{\text{Quantity of 24-hour urine}}{100} = \text{mg. volatile phenols in 24-hour specimen.}$$

B. Determination of Aromatic Hydroxy Acids.—

Transfer the acid solution in the distillation flask to a 250 c.c. separatory funnel, dilute with water to 100 c.c., and shake for three minutes with 30 to 40 c.c. petroleic ether. Draw off petroleic ether and shake twice with ether. Wash the ether with water and evaporate on the steam bath. Add 10 c.c. water before complete drying, then complete evaporation. Use 1 c.c. of this solution which shows a yellow color, with 9 c.c. water. Dilute twice or four times. Use standard phenol solution. The result, multiplied by 1.5, gives the content of aromatic hydroxy acids expressed as mg. p-hydroxy-phenylacetic acid.

*Folin, O., and Ciocalteu, V.: J. Biol. Chem. 73: 627, 1927.

¹Becher, E., Litzner, S., and Täglic, W.: Ztschr. klin. Med. 104: 195, 1926.

²Volterra, M., and Claranfi, E.: Lo Sperimentale 88: 694, 1934.

³Volterra, M.: Am. J. Clin. Path. 12: 525, 1942.

C. Determination of Residual Phenols.—

Dilute the residue from the ether extraction with 200 c.c. water. To 10 c.c. add 1 to 1.5 c.c. silver lactate solution (3 per cent silver lactate in 3 per cent lactic acid) and 1 to 2 drops colloidal iron. Shake, bring to 20 c.c. with distilled water and filter after 15 minutes. To 10 c.c. add 1 to 1.5 c.c. saturated solution sodium chloride in 10 per cent hydrochloric acid. Bring to 20 c.c. with distilled water and filter. Treat 10 c.c. of filtrate with Folin-Ciocalteu reagent as above described. The result, taking into account the various dilutions, gives the so-called residual phenols.

Volterra's normals are as follows:

44.4 mg. volatile phenols in 24 hours; according to this figure on normal subjects, the daily elimination of aromatic hydroxy acids averaged 71 mg.: there was an average of 336 mg. of residual phenols in the normal subject.

HEMOGLOBIN

Hemoglobinuria, or hemoglobin in urine, is caused by dissolution of red blood corpuscles. It is found in severe diseases, such as yellow fever, small-pox, and scarlet fever; following poisoning with carbolic acid, sulphuric acid, naphthol; and after extensive burns. We also have records of cases known as paroxysmal hemoglobinurias. See Teichmann's test, page 1204.

Detection of Hemoglobin in the Urine**Heller's Test**

Precipitate the phosphates from the urine by adding potassium or sodium hydroxide and heating. As they precipitate, they carry with them the hemoglobin, and therefore are blood-red, not white. The coloring matter may be detected microscopically.

Almén's Guaiacum Test

Tincture of guaiacum is made by adding 1 part of guaiacum to 100 parts of absolute alcohol.

Mix equal parts of tincture of guaiacum and old oil of turpentine. This mixture must not have any blue color.

Stratify on this mixture a small amount of urine.

If hemoglobin is present, first a bluish-green, and then a light or dark blue ring appears at the point of contact. On shaking, the mixture becomes blue.

Pus gives a blue ring, but disappears on boiling.

Detection of Hematoporphyrin in Urine

To 100 c.c. urine add 20 c.c. 10% sodium hydroxide.

Collect the precipitate by centrifuging or filtering.

Wash the precipitate with water, then with alcohol.

Add 5 c.c. alcohol and 5 to 10 drops hydrochloric acid, c.p., conc. Mix thoroughly, to dissolve. Filter. Filtrate must be absolutely clear.

Examine spectroscopically for absorption bands of acid hematoporphyrin. (See Chapter XV—Toxicologic Technic for spectroscopic examinations, and Chapter IV—Hematology.)

Porphyria.—

The urine excreted in these cases is Burgundy red with a brown tinge, but sometimes varies from pale pink to almost black. It may be normal yellow when passed but darkens on exposure to light. When the feces contain large amounts of porphyrin, they turn almost black on oxidation.

Acute porphyria is apparently congenital. Patients with this condition show no light sensitivity and porphobilinogen is excreted in the urine in large

amounts. The disease is inherited as a Mendelian dominant and is more common in women than in men. They show the following symptoms: abdominal symptoms, with severe, cramplike pains, vomiting, and pronounced constipation. The abdomen is distended and there may be slight fever and tachycardia. The skin may show a diffuse or spotted pigmentation. The nervous system shows involvement by reason of constant pain in the extremities, accompanied by muscular weakness. There is paralysis in the lower motor neuron type with flaccidity and loss of tendon reflexes. The symptoms suggest Landry's paralysis. Mental symptoms may suggest hysteria. Some patients show toxic delirium with hallucinations of vision and hearing. The urine is dark but may contain only the colorless porphyrinogen.

Hematoporphyrin is found in urine following the administration of such drugs as quinine, tetranol, and others.

Refer to pages 549 and 551 ff. for a further discussion of porphyrins.

Test for Porphyrins.—

Porphyrins may be detected by their fluorescence in ultraviolet light or by means of their absorption spectra.

Reagents.—

Acetic Acid, Glacial, c.p.—

95% Ethyl Alcohol.—

Ethyl Ether, Anhydrous, c.p.—

Hydrochloric Acid, c.p., Concentrated.—

5% Aqueous Hydrochloric Acid Solution.—

Add 5 c.c. concentrated hydrochloric acid to enough distilled water to make 100 c.c. solution.

10% Sodium Hydroxide Solution.—

Use 10 gm. sodium hydroxide per 100 c.c. Keep covered.

Technic.—

Place 25 c.c. urine in a separatory funnel.

Add 10 c.c. glacial acetic acid.

Extract twice with 50 c.c. of ether.

Combine the extractions and wash with 10 c.c. of 5% hydrochloric acid.

Examine the washings under ultraviolet light.

A red fluorescence indicates coproporphyrin. The urinary residue under ultraviolet ray shows a red fluorescence if urinary porphyrins are present.

Confirmatory Test.—

Add 20 c.c. of 10% sodium hydroxide to 100 c.c. urine.

Filter and wash the precipitate with distilled water.

To the precipitate on the filter paper add 5 drops of concentrated hydrochloric acid.

Wash into a test tube with the addition of 5 c.c. of 95% alcohol.

Examine the filtrate spectroscopically for absorption bands of hematoporphyrin.

Test for Coproporphyrins and Uroporphyrins

Method of Ham¹

1. Examine the urine in a dark room. Expose to ultraviolet light for fluorescence, which indicates the presence of porphyrin.

2. **Separation of Coproporphyrin.—**

Technic.—

Acidify 100 c.c. of urine with 10 c.c. of glacial acetic acid and allow to stand exposed to the air overnight.

¹Ham, G. H.: Syllabus of Laboratory Examinations, Cambridge, Mass., 1950, Harvard University Press.

Place in a separatory funnel and extract 3 times with ethyl ether. Use 1 to 2 times the volume of the original mixture for each extraction.

Combine the ether extracts and wash once with 50 c.c. of distilled water.

Return the distilled water washing to the original urine sample.

Extract the *ether extract* 3 times with 2 c.c. of aqueous hydrochloric acid, 25% by volume.

Cool the hydrochloric acid extracts and examine under ultraviolet light.

The Urotype Porphyrins:

Adjust the reaction of the previously extracted urine-acetic acid mixture to pH 3.0 to 3.2 by adding either aqueous hydrochloric acid (1% by volume) or solid sodium acetate.

Extract the urine with solid ethyl acetate, using 1 or 2 times the volume of the urine for each extraction.

Combine the ethyl acetate extracts and wash once with 50 c.c. of distilled water and save.

Extract the ethyl acetate extract further 3 times with 2 c.c. amounts of 25% hydrochloric acid.

Pool the acid extracts and examine for fluorescence and for the absorption spectra.

Screening Test for Porphobilinogen in the Presence of Urobilinogen and Indol Derivatives

Method of Watson and Schwartz²

Reagents.—

Ehrlich's Aldehyde Reagent, Watson's Modification.—

Dissolve 0.7 gm. of para-dimethylaminobenzaldehyde in a mixture of 150 c.c. of concentrated hydrochloric acid and 100 c.c. of distilled water. Store in a dark brown glass bottle.

Saturated Solution of Sodium Acetate.—

Place approximately 1,000 gm. of sodium acetate ($3\text{H}_2\text{O}$) in a liter volumetric flask. Add distilled water to the liter mark and heat to about 60°C .

Allow to cool.

Mix thoroughly.

Technic.—

The urine specimen must be *fresh*.

Mix 2.5 c.c. of fresh urine

with 2.5 c.c. of Ehrlich's reagent in a test tube.

Add 5 c.c. of saturated sodium acetate solution.

Observe for development of a pink color.

Add 3 to 5 c.c. of chloroform.

Shake thoroughly and observe the color of the chloroform layer.

Interpretation.—

If a pink to red color develops after the addition of Ehrlich's reagent and sodium acetate, it may indicate the presence of both urobilinogen and porphobilinogen.

If after extraction with chloroform, the red color remains in the aqueous portion of the tube, this indicates the presence of porphobilinogen, which is insoluble in chloroform.

If the red color is taken out of the aqueous layer and transfers itself to the chloroform layer, it indicates the presence of urobilinogen and no porphobilinogen since urobilinogen is soluble in chloroform.

If the color remains in both layers, it indicates the presence of both porphobilinogen and urobilinogen.

²Watson, C. J., and Schwartz, S.: *Proc. Soc. Exper. Biol. & Med.* 47: 393, 1941.

Reagents.— THE DIAZO TEST OF URINE (EHRlich)

(A) Sulphanilic acid	1.0 gram
Concentrated hydrochloric acid	10.0 c.c.
Distilled water	200.0 c.c.
.(B) Sodium nitrite	0.5 gram
Distilled water	100.0 c.c.
(C) Concentrated ammonium hydroxide.	

Technic.—

Mix 100 parts of solution (A) and 1 part of solution (B).

Place in a test tube equal parts of the above mixture and urine.

Stratify 1 or 2 c.c. ammonium hydroxide on the surface of the mixture. A garnet ring will form at the point of contact if the test is positive.

Shake the tube thoroughly. The foam should become a distinct pink color. The color varies from eosin color to deep crimson, depending on the intensity of the reaction. Any trace of yellow or orange should be regarded as a negative reaction, since the color of a true positive is decidedly pink. A doubtful reaction is considered negative.

Typhoid Fever.—Positive in practically all cases on about the fourth or fifth day. A positive in dilutions of 1:50 and higher is extremely significant in typhoid fever. Mild typhoid cases are negative at this dilution. The reaction begins to fade after two weeks, then becomes negative. Early negative is favorable. The reaction reappears during a relapse, but is negative in a complication.

Tuberculosis.—The test is not diagnostic, although a continued positive in pulmonary tuberculosis is considered grave. It appears intermittently until death. It may be present in mild cases with febrile complications.

Measles.—A positive reaction is present in measles but not in German measles. It appears before the eruption and persists five days.

DETERMINATION OF DRUGS IN URINE

(See Chapter XV for a discussion of the examination of urine for drugs.)

Certain drugs alter the color of urine (see Chapter XV, Toxicology).

Dark green: phenol, salol, creosote, resorcinol

Red: antipyrine, trional

Brown or black: pyrogallol

Yellow: phenacetin, picric acid; santonin changes from bright yellow to scarlet with sodium hydroxide.

Iodine.—Acidify the urine with hydrochloric acid. Add a few drops of starch solution and a few drops of calcium chloride. In the presence of iodine, a blue color develops.

Carbolic Acid.—If carbolic acid is present, the urine becomes dark on standing, green-brown to black. In carbolic acid-containing urine, we find sulphuric acid.

Salicylic Acids.—Salol and Salophen. Addition of iron chloride produces a violet coloration. If there is only a trace of these drugs present, acidifying, shaking with equal parts of ether, and then adding diluted ferric chloride will give the same reaction. See Chapter XV, Toxicology.

Antipyrine with Ferric Chloride.—This gives a red color.

Phenacetin.—To a few c.c. of urine in a test tube add two drops of concentrated hydrochloric acid, two drops of 1% sodium nitrite solution, and a few drops of alkaline alpha-naphthol solution. Now alkalinize. A light red color is produced. This becomes yellow on the addition of hydrochloric acid.

Rhubarb and Senna.—The presence of rhubarb and senna is detected by alkalinizing the urine, which gives a deep red color.

Phenolphthalein.—Phenolphthalein, which is a constituent of some purgatives, appears in the urine and can be discovered by the addition of sodium hydroxide solution, producing a red color.

Pyramidon.—Pyramidon in the urine is detected by the addition of tincture of iodine, producing a yellow ring.

Quinine.—Quinine may be detected with the following reagent:

Solution No. 1:—Potassium iodide ----- 10 gm.

Hot distilled water ----- 50 c.c.

Solution No. 2:—Mercuric silver iodide ----- 2.7 gm.

Distilled water ----- 150 c.c.

Solutions 1 and 2 are mixed together and 2.5 gm. of acetic acid are added. The addition of this reagent to an alkaline-free urine in the presence of quinine gives a cloudiness. As albumin will also cloud, it should be eliminated. The cloudiness produced by quinine disappears on heating, while the cloudiness produced by albumin remains.

Quinine in Urine—A Simple Field Test¹

Reagent.—

Mercuric iodide----- 3.0 gm.

Potassium iodide----- 2.0 gm.

Glacial acetic acid----- 20.0 c.c.

Distilled water, sufficient to make 60 c.c. of reagent.

The reagent must be kept in an amber bottle.

Technic.—

One or 2 c.c. of urine are used for a test.

To a small amount of urine (1 or 2 c.c.) add 5 to 10 drops of reagent.

An immediate turbidity which appears on slight agitation is an indication of quinine. This intensifies upon standing. This method will detect as little as 0.001 mg. of quinine.

Substances such as atropine, cocaine, strychnine, atebrian, and albumin interfere with the reaction. The presence of the alkaloids would be rare; if the patient has taken these drugs, this fact would likely be known. Albumin may be more frequently encountered; its presence may give a false positive. In a suspected false positive, a simple differentiation may be made by heating the tube. The quinine precipitate will dissolve with heat and reappear when cooled. The albumin precipitate intensifies when heated and persists.

This test may be of some value in determining whether the patient has been taking and absorbing quinine. It will detect ingestion of a 5-grain tablet of quinine up to the 36th hour.

Sulfonamides

Refer to pages 499 ff. for methods of testing for sulfonamide drugs.

Antibiotics

Refer to Volume II, Chapter X, Bacteriology, for a discussion of the antibiotics.

Arsphenamine in Urine

Dicken's Modification of Autenrieth's Test for Arsenic

Reagents.—

Concentrated Hydrochloric Acid.

0.5% Sodium Nitrite: Made by dissolving 0.5 gram sodium nitrite in 100 c.c. distilled water.

10% Resorcinol: Made by dissolving 10 grams resorcinol in 100 c.c. distilled water. It should be colorless.

20% Sodium Carbonate Solution: Made by dissolving 20 grams anhydrous sodium carbonate in 100 c.c. distilled water with heat, and filtering through filter paper.

¹Cornell, V. H., and Kaye, S.: *Military Surgeon* 93: 2, 1943.

Technic.—

To 10 c.c. urine in a test tube add 3 drops of hydrochloric acid. Allow to stand five minutes.

Add 10 drops of 0.5% sodium nitrite solution. Allow to stand three minutes.

In another test tube, place 5 c.c. 10% resorcinol, and 3 c.c. of 20% sodium carbonate solution.

Overlay the resorcinol solution with the diazotized urine.

In the presence of arsphenamine a rose-red ring develops slowly at the point of contact of the two liquids. In the presence of atoxyl, a yellow ring develops. The lower portions of the test tube are used as controls. Mix the contents thoroughly. The color should remain.

OCCULT BLOOD IN URINE**The Phenolphthalin Test for Occult Blood¹****Method of Gettler and Kaye****Phenolphthalin Reagent.—**

Place 200 c.c. of distilled water

20 grams of sodium hydroxide

1 gram of phenolphthalein in a 500 c.c. Erlenmeyer flask.

When completely dissolved, add 20 grams of granulated zinc (20 to 30 mesh).

Using a reflux condenser to prevent evaporation, slowly boil the mixture until the red color of the alkaline phenolphthalein disappears, leaving a colorless or faintly yellow solution. This may take two to three hours. The phenolphthalein is thereby reduced to phenolphthalin which is colorless in alkaline solution.

When cool, place in a brown bottle and stopper with a rubber stopper. Some of the zinc may be added; this will aid in keeping it in the reduced form. The reagent will keep for many months if kept in a cool dark place.

Technic.—

Place 1 or 2 c.c. portions of urine, or spinal fluid, or thin fecal suspension, or gastric contents in a test tube and boil for about 30 seconds in order to destroy any oxydases that may be present.

Cool and add about 5 drops of the phenolphthalein reagent, followed by about 3 drops of 3 per cent hydrogen peroxide.

A pink to red color indicates the presence of blood. The intensity of the color is a rough indication of the quantity of blood present. The color may fade after three minutes.

Tests for blood in feces and in gastric contents should be applied only if the patient has been on a meat-free and fish-free diet for three to four days. Bleeding, as seen in hemorrhoids, as well as the admixture of menstrual blood, is to be considered in the interpretation of the results.

The phenolphthalin test is slightly more specific for blood than are the guaiac, benzidine, and orthotolidine tests, and is especially recommended for medicolegal work.

Comparison of the sensitivity of the four tests was found to be:

guaiac test	-----1 part in 10,000
benzidine test	-----1 part in 1,000,000
orthotolidine test	-----1 part in 10,000,000
phenolphthalin test	-----1 part in 10,000,000 (if sample is fresh)
	1 part in 1,000,000 (if sample is old and decomposed)

Benzidine Test

Follow the method given on page 1204, substituting urine for gastric contents.

¹Gettler, A., and Kaye, S.: Am. J. Clin. Path. 13: 9, 1943.

Benzidine Dihydrochloride Test

This test has the advantage over the benzidine test just described in that it is used in aqueous solution, and the solution is stable for at least three months.

Reagents.—

1% Aqueous Solution of Benzidine Dihydrochloride*

Shake 1 gm. of benzidine dihydrochloride and 100 c.c. distilled water at intervals for about 2 hours, or until dissolved. Filter and keep in a dark amber bottle. Do not use heat in preparing the solution.

3% Hydrogen Peroxide Solution

Dilute 30% hydrogen peroxide, c.p., with distilled water in the proportion of 1 volume of 30% peroxide to 9 volumes of water.

1% Aqueous Solution of Sodium Acetate

Dissolve 1 gm. sodium acetate crystals (reagent) in enough distilled water to make 100 c.c. total solution.

Detection of Blood in Urine.—

Place 1 or more c.c. of urine in a clean test tube and dilute to about 5 c.c. with distilled water.

Add, in the order named

1 c.c. of benzidine dihydrochloride solution.

1 c.c. hydrogen peroxide solution

1 c.c. sodium acetate solution.

A blue color develops in the presence of blood. The rapidity with which the color develops depends chiefly on the amount of blood present, from 1 to 10 seconds usually being required.

When the amount of blood is very small, centrifuge the urine, pour off the supernatant fluid, and place the sediment on a glass slide. Add, in the order named, one drop of the benzidine dihydrochloride solution, one drop of the hydrogen peroxide solution, and one drop of the sodium acetate solution, and mix by tilting the slide.

Detection of Blood in Feces.—(See Chapter VIII.)

Mix about 1 gram of feces in a test tube with 5 c.c. of distilled water, bring to a boil over a flame, and filter. Collect the filtrate in a clean test tube, and cool. Add, in the order named, 1 c.c. of the benzidine dihydrochloride solution, 1 c.c. of the hydrogen peroxide solution, and 1 c.c. of the sodium acetate solution. Color reactions are the same as when urine is used in the test.

The Gregersen Modification of the Benzidine Test is described on page 1292.

Orthotolidine Test, Stone and Burke¹

Orthotolidine² was first proposed by E. B. Phelps³ in 1909 as a qualitative test for minute amounts of free chlorine or hypochlorites in water. It is a crystalline basic body of the aromatic series obtained by reduction from orthonitrotoluene, having a melting point of 129° C. and is quite insoluble in distilled water, but soluble in acid solutions and in alcohol and ether. Ruttan and Hardisty⁴ in 1912 reported the use of orthotolidine as a test for the detection of blood, using a 4 per cent solution in glacial acetic acid with a small amount of Perhydrol (Merck) as an oxidizing agent to obtain a blue color in the presence of blood.

*Merck & Co., Rahway, N. J.

¹Stone, W. J., and Burke, G. T.: J. A. M. A. 102: 1549, 1550, 1934.

²Orthotolidine can be obtained from the Research Laboratory, Eastman Kodak Company, Rochester, N. Y.

³Phelps, E. B., quoted by Theriault, E. J.: Pub. Health Rep. 42: 668, 1927.

⁴Ruttan, R. F., and Hardisty, R. H. M.: Canad. M. A. J. 2: 995, 1912.

Stone and Burke reported that they had found orthotolidine useful in the detection of minute quantities of blood in urinary sediments. They point out that red cells in urinary sediments are often crenated or disintegrated in specimens a few hours old and may be confused with leukocytes. Red cells are frequently disintegrated in alkaline urine. The continued presence of red blood cells in urinary sediments has important clinical significance by attracting attention to glomerular inflammatory changes, which are frequently overlooked in the absence of albuminuria or other gross evidences of disturbance. Stone and Burke have found that red cells were excreted more or less constantly in patients with evident foci of infection indicating evidences of glomerular inflammation. It is well known, of course, that a few red cells, leukocytes, or casts, in the absence of other symptoms, may have little significance following unusual exercise or exertion. The persistent excretion of 1,000 red cells per cubic centimeter of urine would not be considered within the limits of normal variation and would point toward some source of chronic glomerular irritation. This quantity of red cells is not recoverable easily by centrifugation, since disintegration or solution frequently has occurred. From 50 to 80 per cent of red cells are recoverable.

Reagents.—

Orthotolidine, 1%, in chemically pure methyl alcohol. (It dissolves with slight difficulty and keeps at least ten months.)

Acetic Acid.—Hydrogen Peroxide Mixture.

Glacial acetic acid, 1 part, and commercial hydrogen peroxide, 2 parts. (This keeps for three or four months, probably longer.)

Technic.—

Centrifuge 15 c.c. of urine at 1500 r.p.m. for 5 minutes.

Pour off the supernatant fluid.

Prepare a portion of the sediment for microscopic examination in the usual way.

To the remaining sediment, add 2 drops of the orthotolidine solution and 2 or 3 drops of the acid-peroxide solution.

In the presence of blood cells aggregating 100 per cubic millimeter of sediment (approximately 1,350 per cubic centimeter of urine) a greenish blue color develops, lasting about one minute. In the presence of from 300 to 500 red cells per cubic millimeter of sediment (approximately 4,000 to 6,500 cells per cubic centimeter of urine) a deeper blue color develops lasting about one minute. In the presence of larger numbers of red cells, aggregating 1,000 per cubic millimeter of sediment (approximately 13,000 per cubic centimeter of urine) as in hemorrhagic Bright's disease (glomerulonephritis) a deep blue color develops lasting two minutes or longer.

Undiluted blood serum, 10 per cent sodium hydroxide, strong trisodium phosphate solutions, and probably other strong alkalies will give positive reactions. Pus cells or any of the common organic or inorganic constituents found in the urine do not give positive reactions.

This method serves to give an approximate quantitative determination of the number of red blood cells in the urine. Specimens of urine containing as high as 5,000 red cells per cubic centimeter will in most instances be undetected by the usual microscopic examinations, since such numbers may represent only one or two cells per high-power field. It is therefore exceedingly advantageous to use the orthotolidine test to cover all these conditions.

RAPID TABLET TEST METHODS FOR LABORATORY PURPOSES

Tablet test methods for the detection of blood, acetone, albumin, sugar,* etc., in urine have been found quite useful in clinical practice.

Hematest (for occult blood) (Ames Company, Inc.).—

Principle.—

The Hematest tablet reagent employs a modification of the method of Ruttan and Hardesty.¹ Methods for the detection of occult blood are based on the property of the peroxidase of the blood cells to reduce peroxide and liberate oxygen. This oxygen is detected in an acid medium by sensitive indicators, such as benzidine, phenolphthalein, and in the test given here, orthotolidine.

Orthotolidine and strontium peroxide are compressed with tartaric acid and calcium acetate into a tablet. Oxygen which is liberated from strontium peroxide when reduced by peroxidase is detected by the orthotolidine; tartaric acid and calcium acetate supply the necessary acidity.

If the Hematest tablets are kept in amber glass bottles tightly closed, they remain stable for more than 12 months.

Technic.—

Specimens of sputum, urine, and other body fluids may be used in solution. Feces, vomitus, and other bulky materials should be suspended in water.

Place one drop of the specimen fluid on a piece of filter paper.

Put a Hematest tablet in the center of the moist area. Then drop 2 drops of water on the tablet so that they run down onto the paper. If a significant amount of blood is present, a distinct blue area forms within two minutes on the paper surrounding the tablet; if negative, the paper will not change color even though the tablet may turn slightly bluish. Only the discoloration of the *filter paper* is of significance. If allowed to stand long enough, the tablet itself may turn bluish even though the paper itself does not.

Sensitivity with this method is one part of blood in 20,000, with a definite positive reaction within two minutes. Approximately 5 minutes are required to detect one part of blood in 100,000.

Acetest (for the detection of acetone) (Ames Company, Inc.).—

Principle.—

This is an adaptation of Frommer's method. Sodium hydroxide and salicylaldehyde are compressed with citric acid, sodium bicarbonate, and sodium sulphite to form a tablet. The sodium hydroxide through its heat of solution provides the heat necessary for the reaction. The tablets do not completely dissolve during the performance of the test but undergo partial decomposition during which, if acetone is present, a definite color change occurs. This test gives a positive reaction for diacetic acid as well as for acetone, but a negative reaction for β -hydroxybutyric acid.

Technic.—

Place the Acetest tablet on a white surface and allow one drop of the specimen to fall on it. A slight effervescence, with formation of bubbles, occurs. If acetone is present, an orange tint, which may deepen to a dark red, appears. Depth of color plus time required may be used as a quantitative measure. If no color changes occur within 3 minutes, the result is negative.

Acetone Test (Denco†) (Denver Chemical Mfg. Company, Inc.).—

This test for acetone is not difficult. Patients are able with this method to test for acetone in their urine without following a complicated technic.

*Tablets and equipment for these tests are procurable from the Ames Company, Inc., Elkhart, Indiana, and Denver Chemical Mfg. Co., Inc., 163 Varick Street, New York 13, N. Y.

†Denver Chemical Mfg. Company, Inc., 163 Varick St., New York 13, N. Y.

¹Ruttan and Hardesty: Canadian M. A. J., Nov., 1912; Biochem. Bull. 2: 225, 1913.

Reagent.—

The acetone test (Denco) is a dry reagent composed of sodium carbonate, ammonium sulphate, and sodium nitroprusside in anhydrous form. In the presence of acetone, the powder will turn some shade of purple, the intensity of the color depending on the amount of acetone.

Technic.—

Place a small amount of the powder on a slide and add a drop or two of urine. The reaction takes place within about one minute. A *trace* of acetone will turn the powder a light lavender and a *larger amount* causes a dark purple color to develop. When *no acetone* is present the powder takes on a grayish-yellow color. To obtain a more accurate estimate of the amount of acetone present, compare with the comparative color chart supplied with the acetone test kit.

The Cargille Scientific Albumin Test Set.*—

This set is a very accurate and rapid method of spot testing for albumin. This procedure does not change in any way the chemistry of the test with sulphosalicylic acid, the reagent for albumin used by leading insurance companies for over twenty years. The set consists of a Cargille spot test plate and Cargille reagent granules (sulphosalicylic acid).



Fig. 17.—The Cargille kit for spot-testing albumin in urine. (Courtesy Cargille Scientific Inc., New York.)

Directions for Use.—

Place one granule on mirror.

With dropper or rod flow two or three drops of specimen over the granule.

Presence of albumin is indicated by turbidity of liquid.

Faint turbidity, after ten seconds, indicates approximately 10 mg. albumin per 100 c.c. Larger percentages yield correspondingly heavier precipitates. Approximate quantitative ratings of albumin can be made after a few tests on specimens containing known amounts of albumin. This spot test promptly eliminates all specimens free of albumin.

Bumintest (for detection of albuminuria) (Ames Company, Inc.).—**Principle.—**

Bumintest reagent tablets contain specially processed sulphosalicylic acid with an effervescent base. The test is a modification of the well-established sulphosalicylic acid methods. The tablets are stable, uniform in composition, nonpoisonous, and noncorrosive.

*Material for this test is manufactured by Cargille Scientific Inc., 118 Liberty Street, New York 6, N. Y.

Reagent Solution.—

Dissolve 4 Bumintest reagent tablets in 30 c.c. distilled water.

Larger quantities of reagent may be prepared in the same proportion.

The solution is ready for use as soon as the effervescence has subsided.

For a bedside test, dissolve 1 tablet in 8 c.c. of distilled water.



Fig 18.—Turbidity testing of urine for albumin showing negative reaction, trace, small amount, medium amount, and large amount. (Courtesy Ames Company, Inc., Elkhart, Ind.)



Fig. 19.—Contact ring test for albumin in urine showing negative reaction, trace, small amount, medium amount, and large amount. (Courtesy Ames Company, Inc., Elkhart, Ind.)

Technic.—

Place 10 drops of urine in a small test tube.

Add 10 drops of the reagent solution. More than 10 drops may be used of both reagent solution and urine, but equal parts of each must be used, and not less than 10 drops each.

Shake the test tube gently.

The presence of albumin is shown by the turbidity of the solution.

Make reading at once. Record as negative, trace, small amount, medium amount, large amount. See Fig. 18.

Since albumin in minute quantity is a normal constituent of urine, a hypersensitive test is not desirable for clinical purposes. The sensitivity of the Bumintest reagent is such that a positive test denotes a pathologic degree of albuminuria. Bumintest reveals a concentration of 10 mg. or more per 100 c.c. of urine.

Clinitest (for determination of urine sugar) (Ames Company, Inc.).—

Principle.—

Each tablet contains anhydrous copper sulphate, anhydrous sodium hydroxide, citric acid, and sodium bicarbonate. The amount of copper sulphate is calculated to fix the peak of color at 2 per cent with a given volume of urine. The sodium hydroxide gives an excess of alkali after reacting with citric acid, and supplies just sufficient heat for completing the reaction. The citric acid is neutralized by the sodium hydroxide, producing heat and forming sodium citrate, which keeps the divalent copper in solution by forming a complex with it. The small quantity of sodium bicarbonate forms the carbon dioxide essential to the proper functioning of the test.

Heat is generated chemically by the tablet as it dissolves in the urine. The amount is constant and adequate. Approximately half of the heat is furnished by the heat of solution of the sodium hydroxide, the other half by the heat of the reaction between sodium hydroxide and citric acid.

The carbon dioxide, released when the tablet dissolves, forms a protective "blanket" above the hot solution, preventing the atmospheric oxygen from coming into contact with the reducing sugar. Shaking disrupts this layer of carbon dioxide, permitting the entrance of oxygen, and thus altering the reading of the test. **Do not shake the tube.**

Technic.—

The amount of fluid in a test must not exceed 1 c.c.

Do not shake the test tube during the reaction period.

Bottles containing the tablets must be closed when not in use. If a tablet is blue, it has deteriorated and must not be used.

(A) Qualitative.—

Place 1 c.c. of urine in a test tube.

Drop in one reagent tablet.

Wait at least 15 seconds after the boiling stops before reading the result. *Negative*, blue to dark greenish-blue; *positive*, dark green, apple green, greenish-yellow, yellow, orange, black, brown, rust red.

(B) Quantitative.—

This method gives reliable estimations up to 2 per cent. It is recommended for bedside use and for use by diabetic patients in the control of their diets and regulation of their insulin requirements.

Place 5 drops or 0.25 c.c. of urine in a test tube.*

Add 10 drops or 0.5 c.c. of water.

Drop one reagent tablet into the diluted urine and wait at least 15 seconds after boiling has ceased before reading.

Results: *Negative*, solution remains blue; *positive*, solution becomes dark green, a pale green, olive green, tan orange, and rust red. The amount of glucose present should be estimated by referring to the standardized color scale enclosed with the tablets. If more than 2 per cent of glucose is present, the solution will pass through the orange color to darker brown or rust red; such urine should be recorded as 4 plus (2 per cent or more) without reference to the scale.

If more than 2 per cent glucose is present, dilute 1 part of the specimen with 3 parts of a negative urine and use 5 drops of this diluted urine in the test. Multiply the end result by 4 to ascertain the amount of glucose.

The Galatest (for sugar in urine).†—**Reagent.—**

Galatest powder is a dry reagent composed of a bismuth salt, sodium hydroxide, and sodium silicate. In the presence of a reducing sugar, the gray or black color which results is caused by the formation of finely divided, partly colloidal, metallic bismuth.

Technic.—

Place a small amount of the powder on a glass slide and add a drop of urine. Compare the color produced with the comparative chart enclosed in the kit in order to obtain the quantity of sugar present. The reaction takes place in about thirty seconds.

*The test has been standardized on the basis of the U.S.P. drops and using test tubes approximately 15 mm. in diameter.

†Material for this test is manufactured by the Denver Chemical Manufacturing Company, Inc., 163 Varick St., New York 13, N. Y.

Galatest reaction is not interfered with by large amounts of albumin, creatinine, uric acid, or urates.

Ictotest for Bilirubinuria.—

The detection of bilirubinuria facilitates the early diagnosis of hepatic disorders because bile appears in the urine before clinical jaundice is evident, and prior to elevation of icterus index or serum bilirubin values. The Ictotest is sensitive to minimal pathologic concentrations of bilirubin in the urine, and can be used routinely.

This reaction is negative in hemolytic jaundice and positive in obstructive and intrahepatic jaundice. It facilitates differential diagnosis in gall bladder disease, pancreatitis, cirrhosis of the liver, hemolytic anemia, and neoplasms of the biliary tract.

Reagent.—

Ictoreagent Tablets: These are manufactured by the Ames Company, Inc., Elkhart, Indiana.

Technic.—

Place 5 drops of urine on a square of special test mat supplied for this test.

Place a tablet on the mat.

Put 2 drops of water on the tablet.

Read within 30 seconds.

When the reaction is positive, the mat *around* the tablet turns purple. Disregard the color on the tablet. The amount of bilirubin is proportionate to the speed of development and the intensity of the color.

When the reaction is negative, there is no color. Ignore any color which develops after 30 seconds.

Always use the test mat which is furnished by the manufacturer.

MICROSCOPIC EXAMINATION OF URINE

Preparation of Sediment

Pour the urine to be examined into a 15 c.c. conical centrifuge tube. Centrifuge at moderate speed for three to five minutes.

Pour off the supernatant fluid. The sediment remains in the tube. Use a dropper with rubber bulb. Shake the tube to mix the sediment. Draw up a drop of sediment in the dropper, and place in the center of a glass slide. Place a clean cover glass over the drop. Take care to eliminate bubble formation.

Make a survey examination under low magnification to detect the presence of casts, and to determine quantities of cells and crystals present. Then examine under high dry magnification to make a close study of the contents of the specimen.

If a bacteriologic examination is ordered, the procedure is different (see Chapter X, Vol. II).

Urinary Sediments

There are two general types of urinary sediments; namely, organized and unorganized.

Organized Sediments

1. Casts: granular, hyaline, epithelial, blood, fatty, waxy, pus.
2. Cylindroids.
3. Epithelial cells.
4. Leukocytes (pus cells).
5. Erythrocytes.
6. Spermatozoa.
7. Urethral filaments.
8. Tissue debris.
9. Animal parasites.

10. Fibrin.
11. Microorganisms.
12. Foreign substances due to contamination.

Unorganized Sediments

1. Ammonium magnesium phosphate (triple phosphate).
2. Calcium oxalate.
3. Calcium phosphate.
4. Calcium sulphate.
5. Calcium carbonate.
6. Uric acid.
7. Urates.
8. Cystine.
9. Cholesterol.
10. Hippuric acid.
11. Leucine, tyrosine.
12. Sulfonamide crystals.

Organized Sediments

Casts

Tubular casts were originally found in the urine by Henle in 1842. In 1867, Roviada gave a thorough account of their nature and formation. Casts are probably the products of an albuminous exudate from the blood vessels with addition of swollen and destroyed epithelia. In almost all cases in which casts are present, albumin is found in moderate or large amount; but there are undoubtedly cases in which the amount of albumin is small, or may be entirely absent.

The appearance of casts in the urine is always of the highest diagnostic importance; their presence probably indicates some kind of nephritis. Hyperemia of the kidneys may suffice to throw casts into the urine, and they can at times be found in small numbers when the kidneys are perfectly intact. They have been described in cases of gastrointestinal catarrh, in jaundice, acute and chronic anemia, as well as in nervous affections of different kinds, without any inflammation of the kidneys. In general it may be said that in the majority of cases the presence of tube casts signifies the presence of nephritis, but small numbers of hyaline casts may be found in renal congestion, unaccompanied by an inflammation.

Casts have been divided in many different ways, but perhaps the simplest is to divide them into *true* casts and *false* casts. The former denote the presence of nephritis, but the latter are accidental formations.

Generally speaking, hyaline, epithelial, and blood casts are found in acute nephritis, while granular, fatty, and waxy casts are found in the chronic types.

All true casts may appear in three distinct sizes, according to the portion of the uriniferous tubules from which they originate. The narrowest casts are those formed in the narrow tubules, the next in size from the distal convoluted tubules, while the largest are always formed in the straight collecting tubules. Casts from the proximal convoluted tubules, those directly arising

from the capsule of the tuft, never appear in the urine, since they cannot pass the narrow tubules. A certain prognostic value attaches to the size of the casts. The mildest degrees of the disease are indicated by casts from the narrow tubules, and a small number of casts from the convoluted tubules. Occasionally, pedunculated casts are found; that is, formations from the place of transition of the narrow tubules into the distal convoluted tubules. Casts from the convoluted tubules justify the diagnosis of chronic nephritis in the cortical substance. Casts of all three sizes, the largest arising from the straight collecting tubules, permit of a conclusion of chronic nephritis in the whole organ, and upon this condition a very unfavorable prognosis can be established.

Repeated examinations of urine, especially from a mixed twenty-four-hour voiding, should always be made before an opinion as to the prognosis of a case can be of any value. All the features, chemical, microscopic, and physical findings must be taken into consideration.

Hyaline Casts.—The hyaline casts are pale, transparent formations of variable length, sometimes of considerable size, and are the most difficult forms of renal casts to detect under the microscope. Those from the convoluted and straight collecting tubules are usually more or less regular, though the latter may be very broad; those from the narrow tubules are occasionally tortuous or spiral, and at times exceedingly narrow and delicate. Different formations, such as pus corpuscles and fat globules, may be seen upon them in small numbers, but are accidental and do not change the diagnosis. When very delicate and pale, it is advisable to color the casts by the addition of a drop of iodine-potassium iodide solution (iodine, 1 part; potassium iodide, 2 parts; water, 300 parts) upon the slide, which will stain them yellow and render them more distinct. (See page 95 for a stain for urinary casts.)

Epithelial Casts.—Epithelial casts, when present, always denote an acute process; the more pronounced it is, the larger is the number of these casts. They vary in size according to their origin, but are never as long as some hyaline casts and are usually quite regular. The number of epithelia seen upon these casts varies considerably. Sometimes no more than two, three, or four will be found upon a cast, while at other times the cast is completely filled with them, though still showing its structure plainly. Those from the convoluted and narrow tubules contain spherical or slightly irregular epithelia, while those from the straight collecting tubules usually contain a number of columnar epithelia. Occasionally these casts are of a yellowish color with a slightly increased refracting power, because of their imbibition of the coloring matter of the blood. As long as the nephritis is acute, the epithelial casts will have this appearance. As soon as the inflammation enters the subacute or chronic stage, their character changes and fat globules appear. We can then no longer consider them pure epithelial casts.

Granular Casts.—Granular casts rarely appear in strictly acute inflammations. They are perfectly regular and have sharply defined contours, or they are more or less curved, or appear curved at one side, while they are straight at the other. Their ends are either rounded or partly broken, and they may be broader at one place and narrower in another—a peculiarity

especially pronounced in those from the narrow tubules. The granular material of which they are composed consists of albumin, epithelial cells, fat, or disintegrated leukocytes or erythrocytes. Their granulations vary from fine to coarse. They may be coarsely granular at both ends and finely granular in the center, or finely granular above and below and coarsely granular in the center. They are due to a disintegration of the kidney epithelia. In cases of long duration the granules become changed into glistening fat granules and globules.

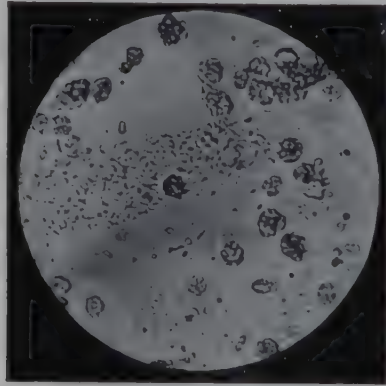


Fig. 20.—Pus cells and finely granular cast. (X400.)



Fig 21.—Urinary casts (Bizzozero). (a) Epithelial cast; (b) and (b') showing nuclei; (c) waxy cast; (d) hyaline cast; (d') convoluted hyaline cast; (e) blood cast; (f) pus cast; (g) hyaline casts with epithelial and white cells; (h) waxy cast with cells. (Klin. Untersuchungsmethoden; Sahli, Vol. II, p. 215. Franz Deuticke, Leipzig, 1931.)

Blood Casts.—The appearance of blood casts in the urine reveals an acute diffuse nephritis, acute congestion of the kidney, or renal hemorrhage. Their appearance varies greatly—they are usually more irregular than the epithelial casts, their ends more or less rounded. They may be studded with a varying number of red blood corpuscles without change in color. If they are retained in the tubules for some time, the blood corpuscles lose their shape, and the

casts take on the appearance of dark, rust-brown, granular clusters. Besides these, conglomerations of fibrin, the so-called fibrin casts, are occasionally found, but, properly speaking, they are not true casts. In the rare cases of hemoglobinuria, irregular, dark casts, which appear granular and are composed of disintegrated blood corpuscles—the so-called hemoglobin casts—may be quite abundant.

Waxy Casts differ in their chemical nature from hyaline casts; they are characterized by wavy, fluted contours, a high refracting power, a more or less yellowish color, and a high degree of brittleness. They vary in size and are always more or less irregular, sometimes resembling regular corkscrew windings. These casts appear only in chronic processes and never in acute inflammations. They invariably indicate amyloid degeneration of the kidney. Pure waxy casts may be found at times studded with different formations, which, of course, do not change the character of the cast. At times they are extremely large, and may then be almost entirely broken in different portions.

Mixed Casts.—Casts in some cases do not appear in their true form, but may be more or less mixed. Any two, three, or four varieties may be so intermingled as to be difficult of differentiation. There may be an epithelial-granular fatty variety, with the epithelia perfectly intact; combinations of waxy casts, fatty and waxy; granular-fatty and waxy; or blood and waxy casts.

Other Casts (Cylindroids).—Besides these varieties, mucus casts or cylindroids are occasionally found among the true casts. They may contain a varying number of fat globules, but their striated, irregular appearance is sufficient to clarify the diagnosis.

False Casts or Pseudocasts have no connection whatever with diseases of the kidney. They are conglomerations of different substances upon mucous threads or casts, or accidental formations in the shape of casts. Among these we designate the urate casts which are conglomerations of urates. Ammonium urate casts are described as occurring in infants, and forming small, reddish-brown masses; they may also be seen in adults, although very rarely. Sodium urate masses, resembling casts, may be mistaken for granular casts; but they have the characteristic yellowish-brown color of sodium urate and show no outlines. We sometimes see a formation composed of granules of sodium urate changing to globules and dumb-bells.

Among the other pseudocasts, the more common are bacterial, pus, fat, and fibrin casts.

Bacterial Casts are of frequent occurrence, especially when the urine has been allowed to stand in a warm room for twelve hours or more, so that a large number of bacteria have developed. They vary considerably in size, but their outlines are pale and more or less irregular. They are composed of masses of micrococci, not of granules. In order to clarify their diagnosis, it may, in rare cases, be necessary to add a drop or two of some strong mineral acid or alkali, to which they will be seen to have a great resistance.

Pus Casts are found in some cases. The pus corpuscles may be massed together, with no outlines visible, or they may be more loosely arranged, and may contain a number of small fat globules. They indicate renal suppuration.

Fat Casts.—Pseudofat casts are rare, but have been found in a few cases of so-called lipuria. They consist of large, fat globules, of a very high refraction, and occasionally contain margaric acid needles.

Fibrin Casts are found in cases of hemorrhage. They are large, have irregular, more or less sharply defined contours, and are of a yellowish or yellowish-brown color.

A Stain for Urinary Casts

The technic of Behre and Muhlberg¹ is very useful in the study of the structure and composition of urinary casts.

Reagents.—

0.5% Aqueous Solution of Eosin Y.—

Dissolve 500 mg. eosin Y in 100 c.c. distilled water.

Methyl Blue-Picric Acid Solution.—

Add 1 c.c. of 1% aqueous solution of methyl blue to 10 c.c. of saturated picric acid solution. Use Merck's reagent picric acid.

Add 10 drops of glycerol to each 10 c.c.²

Technic.—

Centrifuge the urine and decant the supernatant liquid.

Add to the sediment one drop of the eosin solution and mix by shaking from side to side.

After a minute or two add 2 drops of the methyl blue-picric acid solution and mix. The color of the sediment should be distinctly blue green. If it is reddish brown, add more of the blue solution until the blue green color is obtained, but do not add too much.

Transfer some of the stained sediment to a glass slide, cover with a cover glass, and examine under the microscope.

The quantities of the stains added to the urine may be varied according to the amount of sediment. If the cells are not red enough, add more eosin to the sediment. Enough methyl blue should be in the stain to impart a distinct blue color to the casts, but too much stain will make them too dark.

To make permanent mounts, add more glycerol to the sediment and seal the edges of the cover glass with balsam.

By the use of this staining method, hyaline casts are stained a clear blue of varying intensity. The more irregularly shaped bodies, sometimes classed as cylindroids, are similarly stained. An irregular distribution of material, a mealy structure, or a striated appearance sometimes appears in bodies which appear perfectly homogeneous before staining. Mucous threads and amorphous mucous material are also stained blue, making the differentiation from castlike bodies very evident. Mixed, finely granular casts present a striking picture of fine, dark granules powdered over the light blue, hyaline body. Some coarsely granular casts are stained deep blue, the granules of others are yellow, orange, or dark reddish brown. Renal epithelial cells are usually red, sometimes orange or yellowish. Red blood cells are stained a brighter red. Pus cells are usually red, occasionally blue; epithelial cells from the

¹Behre and Muhlberg: J. Lab. & Clin. Med. 22: 853, 1937.

²Instead of methyl blue solution, 1.5 c.c. of blue-black fountain pen ink (Waterman's, Carter's, Skrip, etc.) added to 10 c.c. of saturated picric acid may be used. The dye used in these inks is evidently similar to methyl blue. The results with ink solutions are almost as good as those with methyl blue.

urinary passages are either red or blue. Fat globules are unstained. In cells undergoing fatty degeneration, the fat globules are seen strikingly against reddish cellular material.

By the use of this stain, possible differences in structures otherwise undifferentiated may be made out. In general, the method deserves adoption and further investigation.

It is known from life insurance statistics that casts, either hyaline or granular, and either intermittent or constant, herald the onset of cardiovascular renal disease about ten years before the cardiovascular renal apparatus is seriously affected. Too much attention, therefore, cannot be paid to the microscopic examination of urinary sediment.

Interpretation of Casts in the Urinary Sediment.—The finding of casts of various types in urinary sediment provokes the question of just what significance should be attached to this finding. First, in order to answer the question, we must discuss the types of casts separately. Hyaline casts, for instance, are often found in urine of healthy people after exercise. Their presence does not always parallel the quantity of albumin in cases of renal albuminuria. During recovery from acute nephritis, they may be present for some time after albuminuria has disappeared. Hyaline casts are frequently found in jaundice without any accompanying albuminuria.

The presence of granular casts is always indicative of a severe degenerative process in the tubular epithelium. One may expect, however, to find isolated granular casts without severe renal disease as in chronic passive congestion or febrile albuminuria.

Epithelial casts have the same significance as granular casts; namely, isolated, single casts are of little significance, but a large number indicates renal degeneration.

Fatty casts are found in the later stages of severe acute nephritis and the subacute and chronic stages of the disease, as well as in the nephroses.

Waxy casts are found wherever there are severe degenerative changes in the kidney existing over a long period of time. At one time they were believed characteristic of the amyloid kidney, but this has been disproved.

Hemoglobin casts have been found in the presence of hemoglobinuria.

Cylindroids are found in catarrhal inflammations of the urinary passages, especially in acute disturbances of the renal circulation, such as occur from embolus formation in the renal artery.

Summing up, granular, epithelial, fatty and lipid casts indicate degenerative changes in the tubular epithelium.

Epithelial Cells

Epithelial Cells.—There are three kinds of epithelia: flat, or squamous; cuboidal; and columnar, or cylindrical.

Squamous epithelial cells are large, more or less irregular in outline, exhibiting a broad front surface, while in edge view they are narrower and somewhat spindle-shaped. They are granular, and possess one or more nuclei. Occasionally this drops out, leaving a vacuole.

Cuboidal epithelia have about the same diameter in all directions, while *columnar epithelia* are elongated in one direction. Columnar epithelia may be ciliated, having one or more delicate, hairlike prolongations on the outer surface. All epithelia are granular, and possess one or more nuclei.

Epithelia may occur in a single layer, or stratified. They change in appearance to a certain extent in urine.



Fig. 22.—Epithelia from the vagina. 1, Upper layer; 2, middle layer; 3, deepest layer.



Fig. 23.—Epithelia from the urethra.

Epithelia from the *bladder* are of three distinct types; those from the upper layers are flat, or squamous; those from the middle layers are cuboidal; those from the deepest layer are columnar. Squamous epithelia are insignificant in the urine except when they appear along with pus cells.

Epithelia from the *pelvis of the kidney* are smaller than those from the bladder, but larger than those from the ureters. The majority of the pelvic epithelia are caudate, pear-shaped, or lenticular, although they are sometimes quite irregular. The regular, cuboidal shapes, smaller than those from the bladder, are less numerous.

Epithelia from the *ureters* are rarely found alone, but usually with those from the pelvis of the kidney. They are round, globular, or slightly irregular when seen in urine, and are distinctly smaller than those from the pelvis. They closely resemble the epithelia from the prostate gland. Occasionally small columnar epithelia from the deeper layers are found.

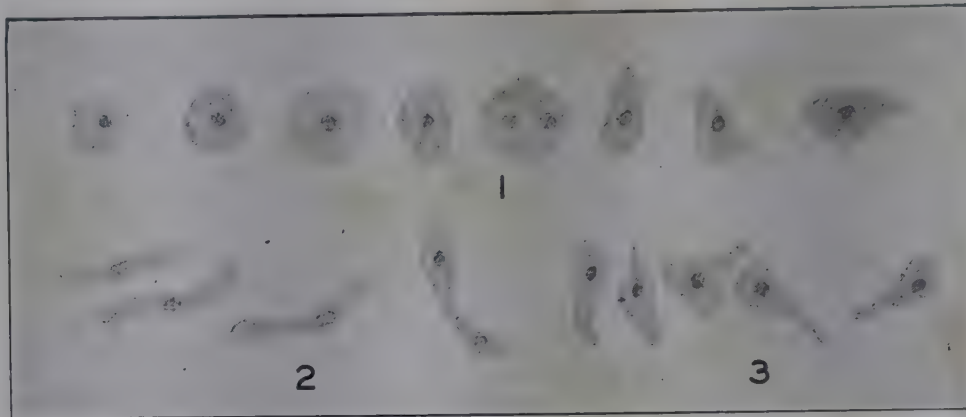


Fig. 24.—1, Epithelia from the prostate gland; 2, epithelia from the ejaculatory ducts; 3, epithelia from the seminal vesicles.

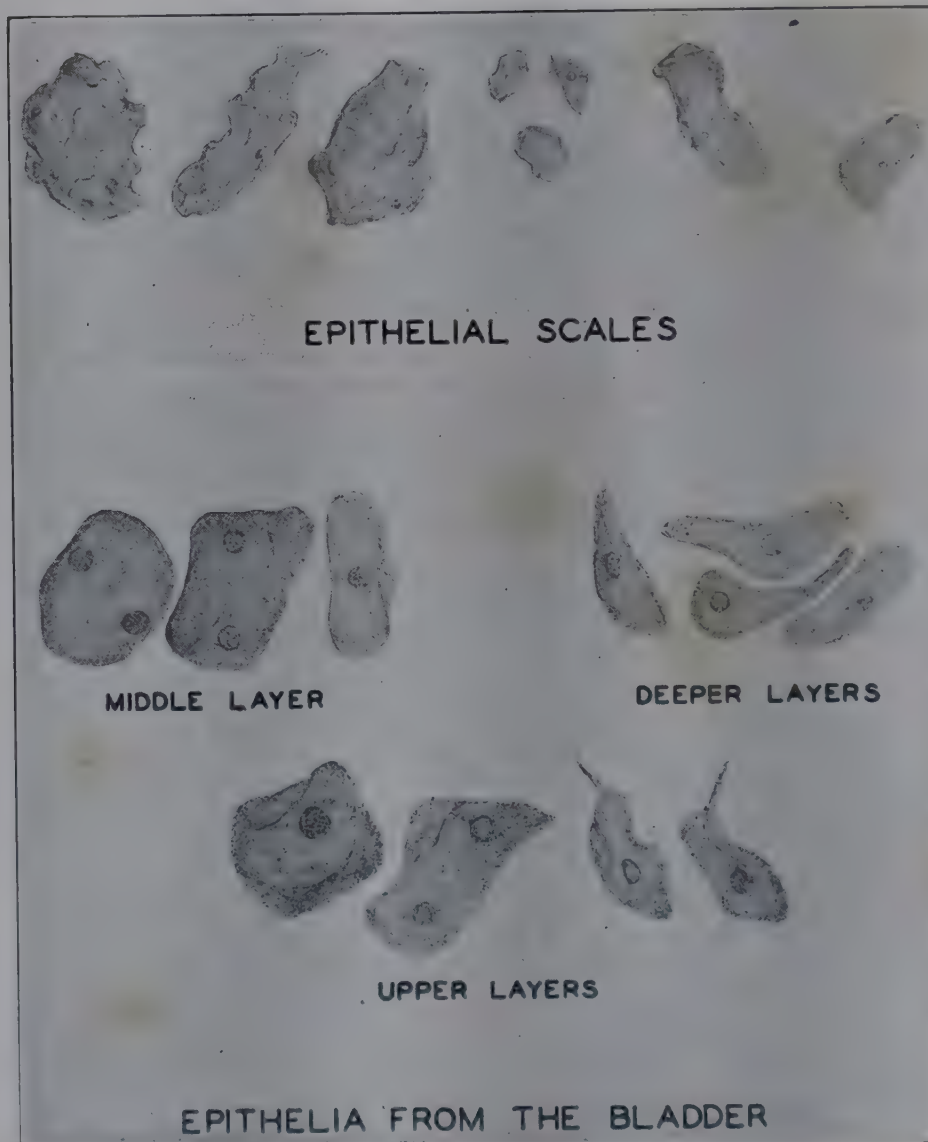


Fig. 25.

Epithelia from the *uriniferous tubules* of the kidneys are very important. They are of two kinds: the cuboidal from the convoluted tubules, and the columnar from the straight collecting tubules. They are distinctly smaller than either those from the pelvis of the kidney, or from the ureter in the

same case, though their sizes vary to a certain degree in different cases. They are round, globular, or slightly irregular. They are about one-third larger than pus cells.

Leukocytes, or Pus Cells, are larger than erythrocytes, and smaller than renal cells. They are about the same size as, or slightly larger than, the nuclei of the large squamous epithelial cells. They contain one or more nuclei which are rendered distinct by the addition of acetic acid.

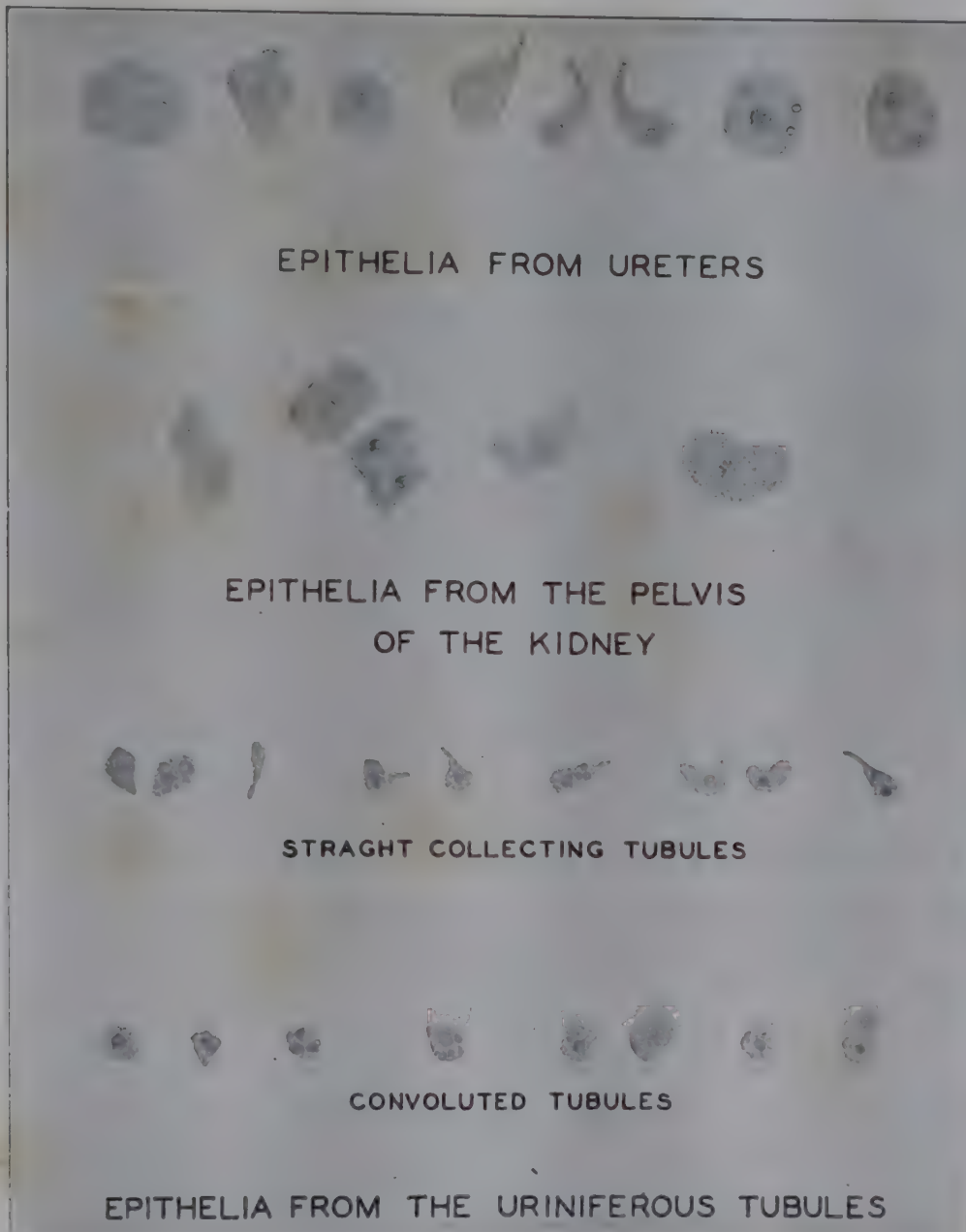


Fig. 26.

Erythrocytes appear in the urine as normal biconcave or crenated disks. They are smaller than pus cells, and have no nuclei. They dissolve in acetic acid. The pathologic conditions in which erythrocytes are found in the urinary sediment are as follows: hemorrhage of the kidney; hemorrhage of the urinary tract; hemorrhage from congestion; traumatic hemorrhage; hemorrhagic diathesis.

Spermatozoa appear after coitus or in the following conditions: diseases of the genital organs; nocturnal emissions; epileptic and other convulsive

attacks. They may or may not be motile. They have an oval body, and a long, delicate tail.

Urethral Filaments.—These peculiar threadlike bodies may be found in normal urines, and also in the following pathologic conditions: acute gonorrhea; chronic gonorrhea; urethrorrhea. These filaments are generally macroscopic. The first morning urine is the best specimen for filaments.

Tissue Debris.—The finding of fragments of tissue may sometimes throw some light upon a pathologic condition. These tissues may be found in the following pathologic conditions: tubercular affections of the kidney; tubercular affections of the urinary tract; tumor of the kidney; tumor of the urinary tract. It is necessary, however, to make a histologic examination of these tissue fragments before coming to a final conclusion as to their origin.

Fibrin.—Fibrin clots are occasionally found in the sediment of urines following hematuria.

Microorganisms.—Fresh urine does not contain any organisms and is usually considered sterile when obtained directly from the bladder, but may be contaminated, when voided, by bacteria present in the urethra or vagina. Bacteria develop in a short time in urine when it is allowed to stand, and are present in large numbers in pathologic conditions. Such urine is turbid. The condition is called "bacteriuria."

Microorganisms in urine may be nonpathogenic or pathogenic. The nonpathogenic microorganisms found in urine are the mold fungi, such as *oidium*, *Penicillium glaucum*, or one of the *aspergilli*.

Yeast fungi are found in acid urine, especially in urine containing sugar. They consist of variously sized globules or cells. In the larger globules, the process of budding can be seen. The most common organism found in urine is the *Micrococcus ureae*. This coccus causes ammoniacal decomposition of urine, urea being transformed by it into ammonium carbonate.

See the chapter on Bacteriology for complete bacteriologic examination of urine.

The fungus known as *actinomyces* is sometimes found in urine. It is the cause of actinomycosis of the internal organs; where the disease affects the genitourinary tract, we find this ray fungus in the urine.

Animal Parasites.—The most common animal parasite found in the urine is the *Trichomonas vaginalis*, a flagellate occurring in the urine of females. It frequently causes symptoms (vaginitis, cystitis). It is an oval organism with a taillike extremity, with rapid motility.

Echinococci are occasionally found in urine and may have developed directly from the urinary organs or may have ruptured into them from some neighboring organ. The characteristic parts of the echinococci found in the urine are the hooklets as well as portions of the membrane. *Scolices* may also be found. These scolices are small, round, and supplied with a wreath of hooklets. When echinococci are found in urine, there is also evidence of hemorrhage or ulceration, or both. Red cells are numerous, together with epithelia and connective-tissue shreds as well as pus corpuscles. The parasite *Schistosoma haematobium* exhibits its eggs in the urine, but never the parasite itself. The eggs are oval, or flasklike, in shape and taper considerably at one

extremity, the other being rounded. The *Filaria* appears in the urine at times, causing severe hematuria or chyluria. Where a milky urine, indicating chyluria, is found, an examination for filaria must be made. In examining urine for filaria, it is advisable to take the first urine voided in the morning, since it is a well-known fact that the parasite is active in the night (*F. nocturna*, or *Wuchereria bancrofti*), or during the resting hours of the patient, while it is quiescent during the working hours and cannot be found.

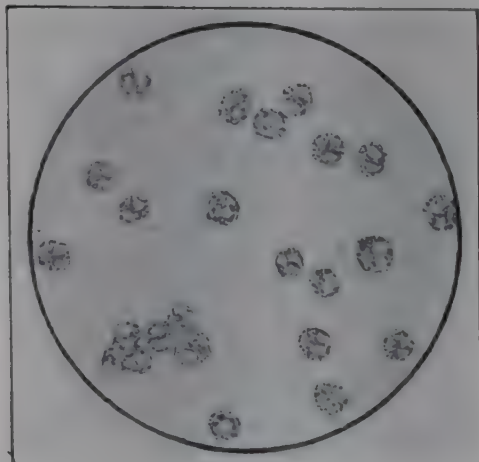


Fig. 27.

Fig. 27.—Pus cells in urine (unstained). (×400.)

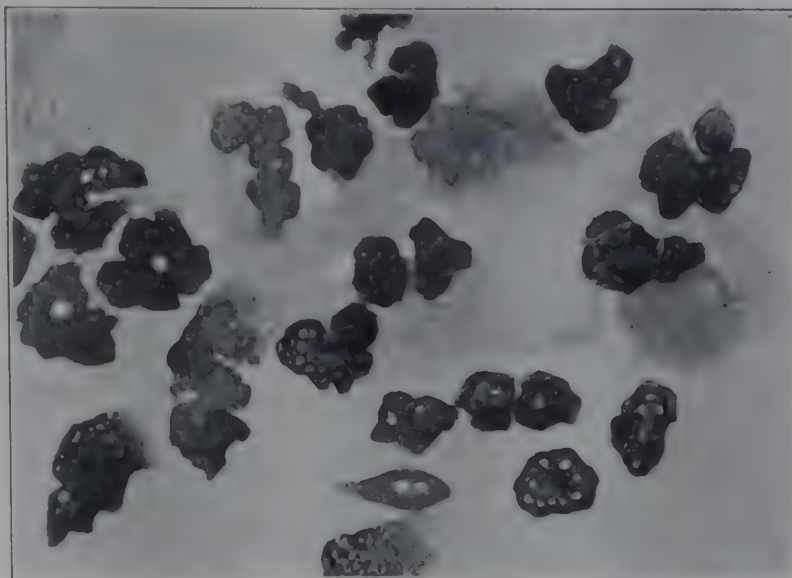


Fig. 28.

Fig. 28.—Pus cells in urine (stained). (×950.)

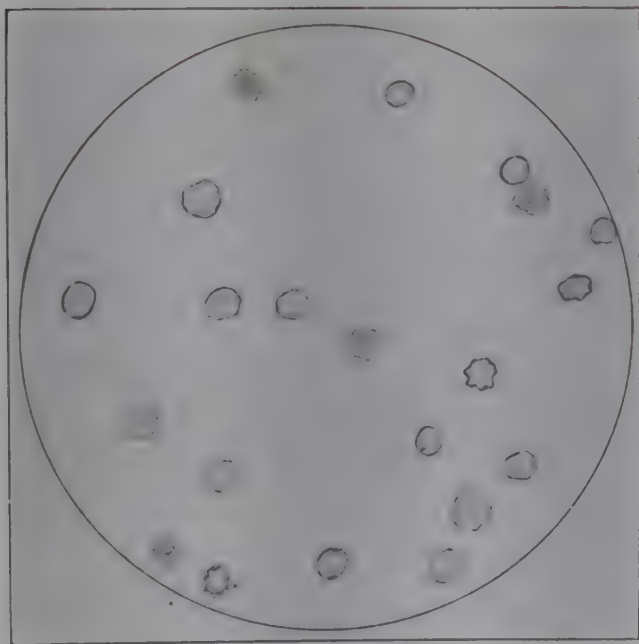


Fig. 29.

Fig. 29.—Erythrocytes in urine sediment (unstained). (×400.)



Fig. 30.

Fig. 30.—Schematic drawing of human spermatozoa (unstained). (×400.)

The *Ascaris lumbricoides*, a common worm in children, may be found in the urine, having passed into the bladder through the urethra. Other parasites which may possibly be found in the urine are the *Strongylus gigas*, *Enterobius vermicularis*, and the *Cercomonas urinarius*.

Refer to Chapter XII for a discussion of parasites.

Foreign Substances in Urine.—Care must be taken that we do not overlook foreign substances in the urine which may be mistaken by beginners for pathologic changes in the body. These substances are starch granules, hair, feathers, muscle fibers, particles of fat, fibers of silk, cotton, wool, etc. The

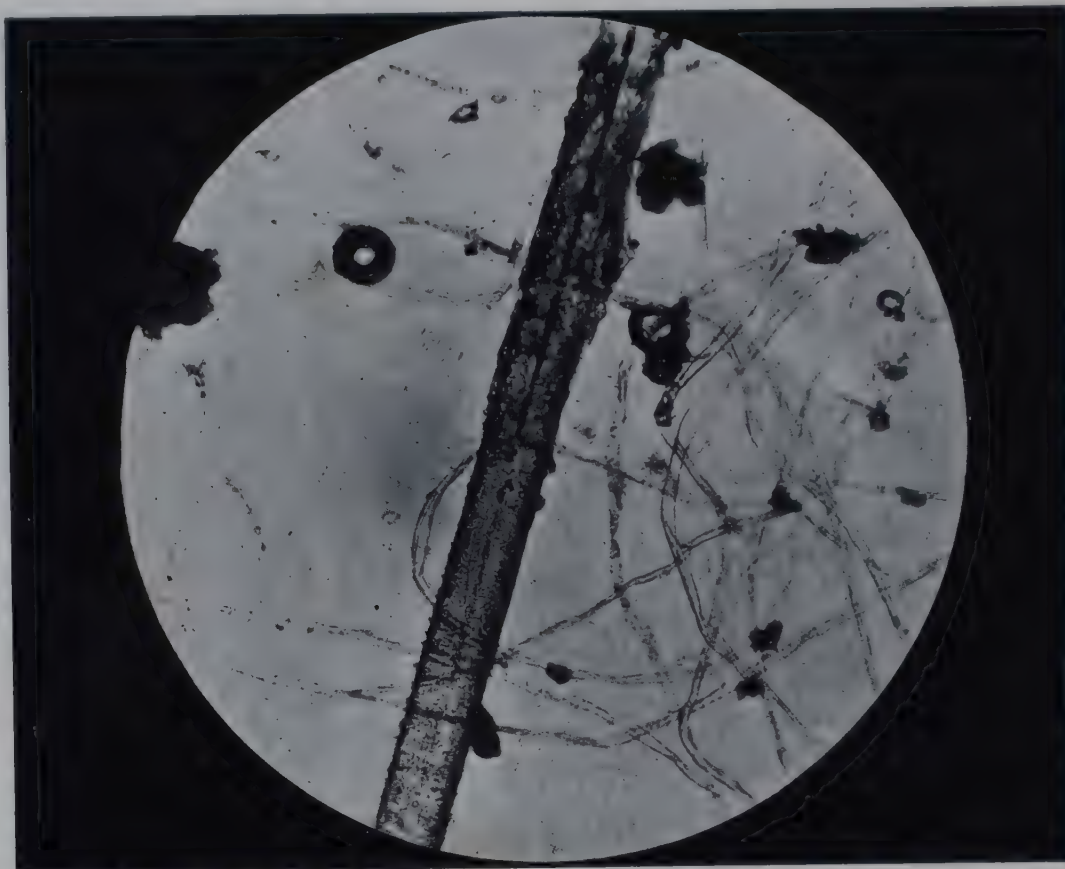


Fig. 31.—Artefacts—hair, air bubble, cotton threads, soot. ($\times 200$.)

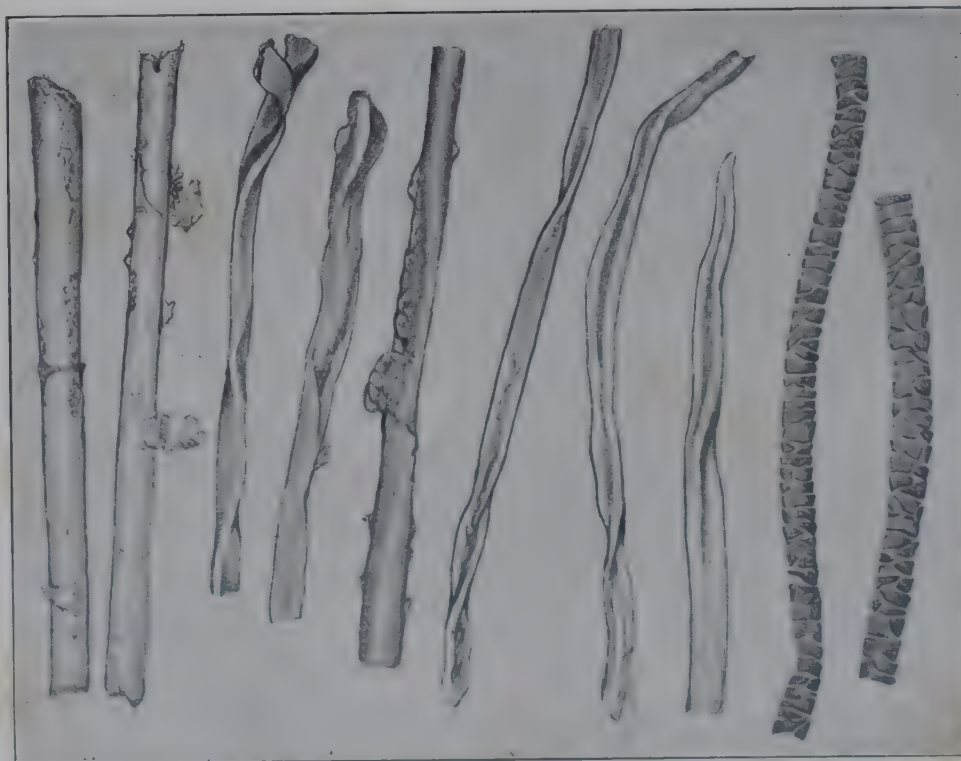


Fig. 32.—Fibers—silk, linen, cotton, wool.

presence of these materials may be due to many causes, such as exposure to air, pouring the urine into bottles which are not perfectly clean, the use of salves or dusting powders for the genital organs, or admixture of particles from the feces. Human hairs are not infrequently found in the urine and

may be known by the flat epidermal scales, firmly attached to each other, which form the main mass of the hair, and by the varying amount of pigment. Feathers may appear in branching formations. Scales from the wings of insects, such as moths, may also be found. They are more or less delicate, serrated plates with a stemlike projection, and vary considerably in length and breadth.

Starch globules are frequently seen in the urine. They are round or oval, highly refractive, and vary greatly in size. The three most frequently found in the urine are rice starch, corn starch, and wheat starch. Rice starch shows oblong, even globules of medium size. Corn starch is smaller, irregular, at times almost hexagonal, and contains an irregular hilum. Wheat starch consists of large globules, as well as of small, irregular formations, in which the hilum may be entirely absent or is present only in the form of a dot.

Lycopodium, similar to starch and used as a dusting powder, consists of globular formations of different sizes, with a distinct shell; these globules are sometimes found in urine. Cellulose occurs in the urine in a variety of forms, sometimes in small, sometimes in large masses. A common variety of cellulose found in urine is cork occurring as single cells and having a yellowish-brown color. Oil-globules and air-bubbles are commonly found in urine. They may be very large or extremely small, and are either perfectly round or irregular, are of high refraction, and can frequently be differentiated by their yellowish color.

Flaws in glass as well as scratches in the cover glass may lead to mistakes in diagnosis. Flaws are irregular in size and shape and frequently resemble the wings of a butterfly. They show a faint blue refraction and are usually pale. Rust particles in both the cover glass and slides also occur and are larger or smaller, dark or rust-brown, irregular masses, which must not be mistaken for coloring matter in the urine. Vegetable matter of different forms may be found in the urine as an admixture from the feces. Normal feces may occasionally be found mixed with urine, and their constituents must be known. If they are present and their accidental admixture can be excluded, the diagnosis of a fistula can be made. Among the particles seen from these sources are: partly digested muscle fibers with many striations plainly visible; small numbers of connective tissue shreds from the meat diet: mucus threads and mucus corpuscles.

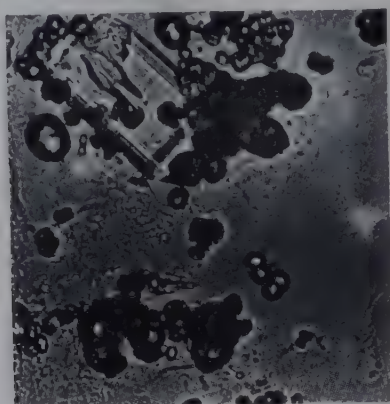
Unorganized Sediments

Crystals

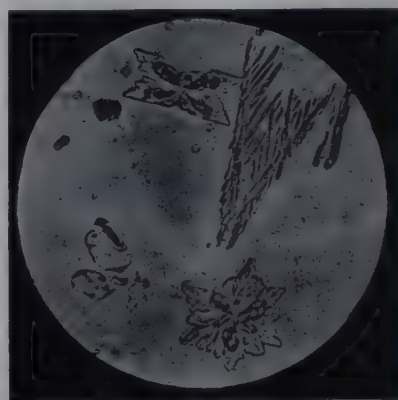
Ammonium Magnesium Phosphate (Triple Phosphate) Crystals are found when the urine has undergone alkaline fermentation, either before or after being voided, and crystallized in two forms: prisms and star-shaped, feathery crystals. These crystals may rarely appear in amphoteric or faintly acid urines, provided the ammonium salts are present in large enough quantity. The pathologic conditions in which these crystals are frequently abundant are as follows: retention of urine in the bladder; paraplegia; chronic cystitis; enlarged prostate; chronic pyelitis.

Calcium Oxalate Crystals appear in the urinary sediment in at least two forms; namely, octahedral type, and dumb-bell type. They may be found in acid, neutral, or alkaline urines, but are most frequently found in acid urines. Calcium oxalate crystals are found in normal urines, but are increased in the following pathologic conditions: diabetes mellitus; organic diseases of the liver; diseases of the heart; diseases of the lungs.

These crystals are found in the urine after ingestion of tomatoes, garlic, rhubarb, oranges, asparagus, etc.



A.



B.

Fig. 33.—A, Triple phosphate and ammonium urate crystals ($\times 400$). B, Incomplete triple phosphate ($\times 400$).

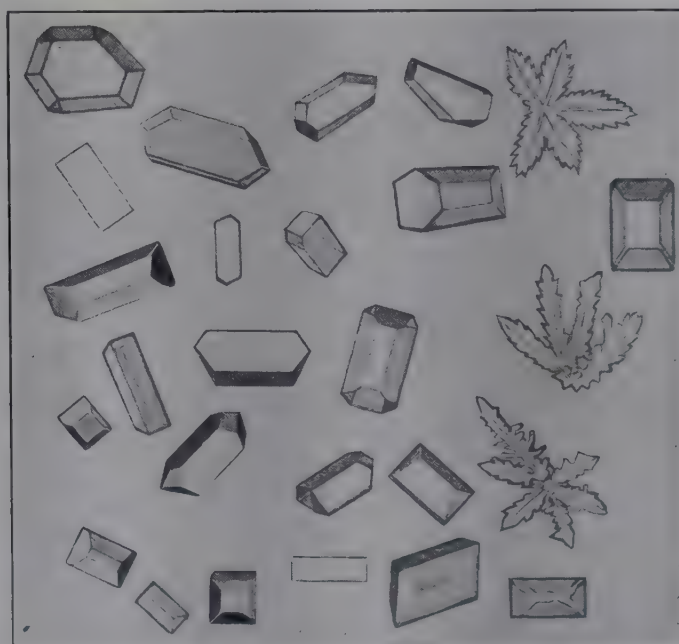


Fig. 34.—Triple phosphate crystals (ammonium magnesium phosphate).

Calcium Phosphate Crystals (Stellar Phosphate) may occur in the urine in the amorphous, granular, or crystalline form. They are wedge-shaped, and often appear in rosette arrangements. They are sometimes mistaken for sodium urate, but may be distinguished from the latter by adding acetic acid. Acetic acid will readily dissolve the phosphate, whereas the urate is much less soluble. The pathologic conditions in which calcium phosphate crystals are abundant are as follows: retention of urine in the bladder; paraplegia; chronic cystitis; enlarged prostate; chronic pyelitis.

Calcium Sulphate Crystals are very rarely seen and are found only in acid urines. They appear as long, thin, colorless needles or prisms and may

be mistaken for calcium phosphate. They are readily distinguished, however, by the fact that calcium sulphate crystals are readily soluble in acetic acid. Calcium sulphate crystals are of practically no clinical importance.

Calcium Carbonate Crystals are almost always in alkaline urine, but may occur in amphoteric or faintly acid urine. They very frequently appear in the dumb-bell shape and can be differentiated from calcium oxalate inasmuch as they dissolve in acetic acid with the evolution of carbon dioxide gas, while calcium oxalate remains unchanged in acetic acid.

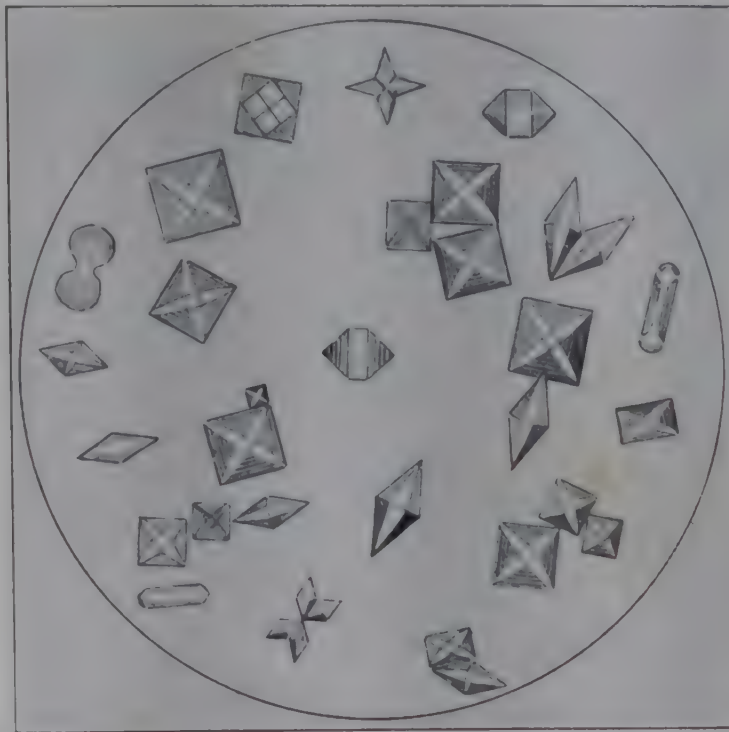


Fig. 35.—Calcium oxalate crystals.



Fig. 36.

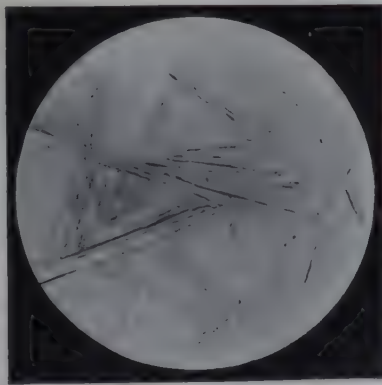


Fig. 37.

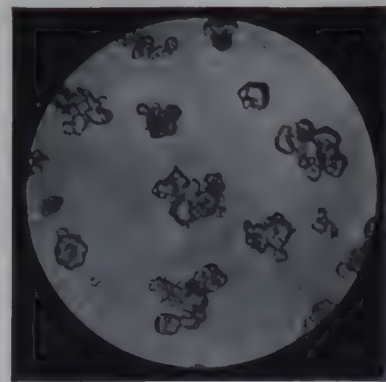


Fig. 38.

Fig. 36.—Calcium phosphate crystals ($\times 400$).

Fig. 37.—Calcium sulphate crystals ($\times 400$).

Fig. 38.—Calcium carbonate crystals ($\times 400$).

Uric Acid Crystals appear in acid urines in the following forms: wedge-shaped; dumb-bells; rhombic prisms; whetstones; prismatic rosettes; irregular or hexagonal plates.

They generally appear in the urine colored brownish-red, although occasionally they are colorless. The presence of uric acid in the urinary sediment does not necessarily indicate any pathologic condition, neither does it mean

that the uric acid content of the urine is increased. The pathologic conditions in which uric acid is found in the sediment are as follows: gout; acute febrile conditions; chronic nephritis.



Fig. 39.—Uric acid crystals.



Fig. 40.—Amorphous urates.

Urates may appear as ammonium, calcium, magnesium, potassium, and sodium urate. The calcium, magnesium, potassium, and sodium urates appear in acid urines, while the sediment of ammonium urate appears in neutral, alkaline, or acid urines.

Sodium Urate may be amorphous or crystalline. When crystalline it appears in sheaves or clusters of colorless needles.

Ammonium Urate generally appears in the burrlike form of the "thorn-apple" which appears to be balls with spicules attached. The pathologic conditions in which urates may appear in the urine are somewhat similar to those of uric acid.



Fig. 41.—Ammonium urate crystals. (After Peyer.)



Fig. 42.—Cystine crystals.

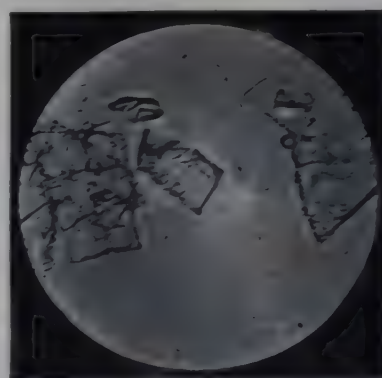


Fig. 43.—Cholesterol crystals ($\times 200$).

Cystine Crystals are rarely found in urinary sediments but may produce concretions in the bladder. Cystine is insoluble in water, alcohol, and acetic acid, and soluble in minerals, hydrochloric acid, alkalies, and especially in ammonia. This solubility in ammonia distinguishes it from uric acid. Cystine contains considerable sulphur. Cystine occurs in all members of certain families instead of uric acid, and in such families it appears to replace uric acid; in them cystine calculi are not uncommon. Cystine consists of hexagonal, colorless plates of moderate sizes, of high refraction, which, in side view, present one perfect facet and two imperfect neighboring facets. A number of plates may lie together, one upon another, or they may form more or less regular masses.

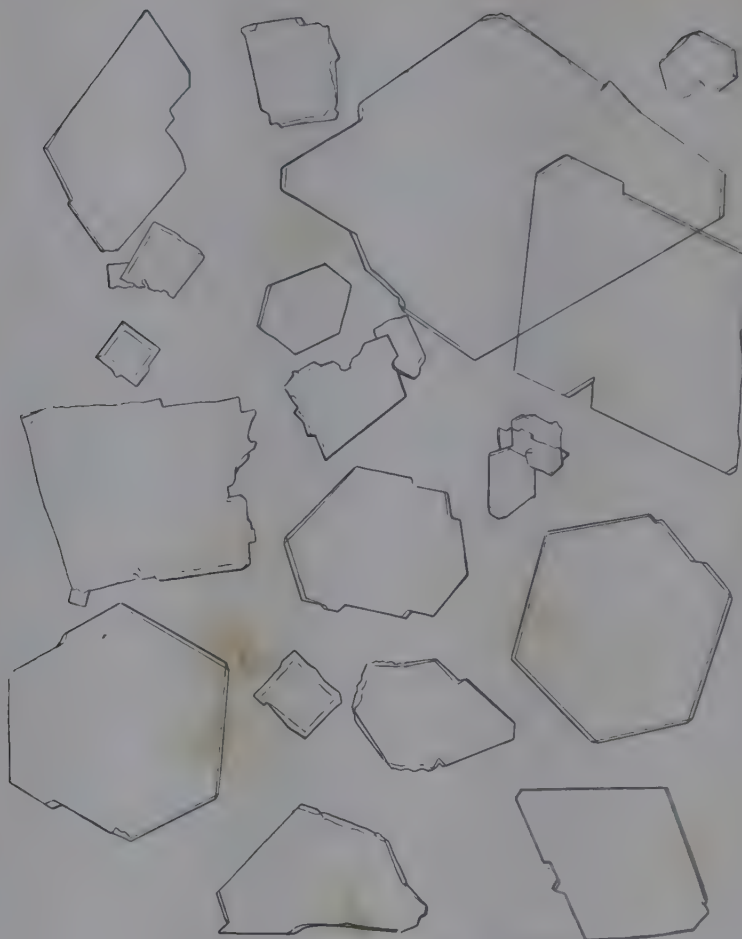


Fig. 44.—Cholesterol crystals.

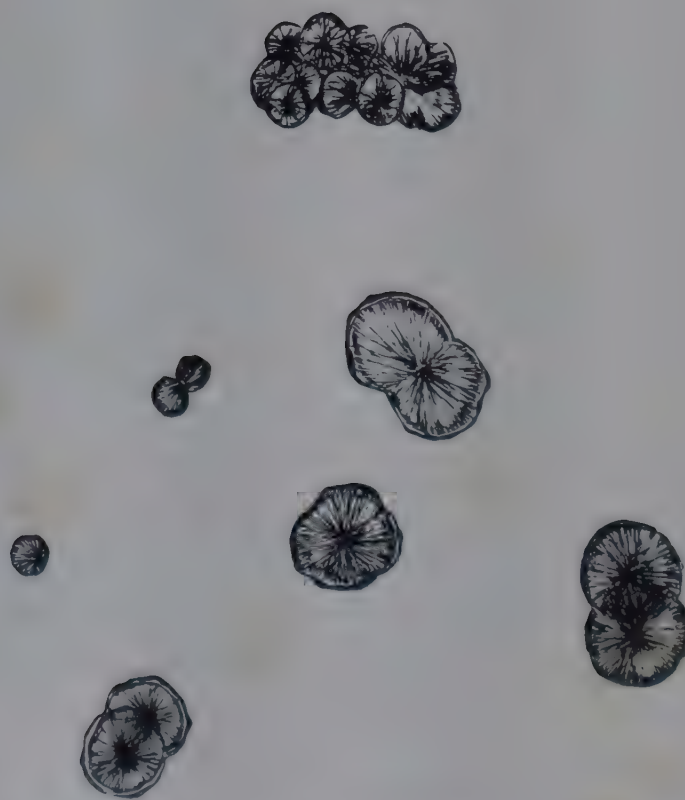


Fig. 45.—Crystals of impure leucine. (After Ogden.)

Cholesterol crystals are very rarely found in urinary sediments. They ordinarily crystallize in regular and irregular, colorless plates which are transparent. They may occasionally be found as a film on the surface of the urine instead of in the sediment. The pathologic conditions in which cholesterol crystals have been found in the urine are as follows: cystitis; pyelitis; chyluria; nephritis.

Hippuric Acid Crystals are very rarely found in urinary sediments; they appear as needles or prisms which are generally pigmented like uric acid crystals; are more soluble in water and ether than uric acid crystals, and have practically no clinical significance.

Leucine and Tyrosine Crystals almost always appear in the urine together, in solution or as a sediment. Leucine crystallizes in characteristic spherical masses and is highly refractive. Tyrosine crystals appear as sheaves of very highly refractive needles.

The pathologic conditions in which leucine and tyrosine have been found are: acute yellow atrophy of the liver; acute phosphorus poisoning; cirrhosis of the liver; severe cases of typhoid fever; severe cases of smallpox; leukemia.

Morphology of Urinary Crystals Appearing During Administration of Sulfa Drugs

The literature on the specific appearance of crystals produced during administration of sulfa drugs is somewhat scant. Lehr and Antopol* have made a study of this question on 260 individual urinary specimens from 175 patients to whom these various drugs had been administered. The drugs studied were sulfapyridine, sulfathiazole, sulfadiazine, and sulfaguanidine. They state that the frequency and quantity of these crystals in the urine are apparently related to the solubility of the sulfanilamide compounds and their acetylated compounds.^{1, 2} The comparatively insoluble acetyl derivatives formed in the body from sulfapyridine, sulfathiazole, and sulfadiazine, if analyzed as urinary crystals, show a depressed melting point and a solubility which is about twice as high as that of the chemically acetylated drugs. This is true, too, of artificially produced urinary crystals. When one recrystallizes these substances from water, the shapes of these crystals and their melting points and solubility values approach those of the synthetic compounds. Thus, this morphology is dependent upon the "impurity" contained in these substances and apparently in many urines of patients receiving these drugs. Repeated recrystallization of the acetyl derivatives removes this "impurity."

The shape of urinary crystals derived from these drugs points to the influence of colloids upon crystal formation.³ These colloids appearing in the urine

*Lehr, D. and Antopol, Wm.: *Am. J. Clin. Path.* 12: 200, 1942.

¹Garvin, C. F.: *J. A. M. A.* 116: 300, 1941.

²Flippin, H. F., Rose, B. S., Schwartz, L., and Domm, A. H.: *Am. J. M. Sc.* 201: 585, 1941.

³Ord, W. M.: *The Influence of Colloids Upon Crystalline Forms and Cohesion*. Edward Stanford, London, 1879. Also Schade, H., in Alexander, J.: *Colloid Chemistry, Theoretical and Applied*, Vol. II, p. 803, Concretions. The Chemical Catalogue Company, New York, 1928.

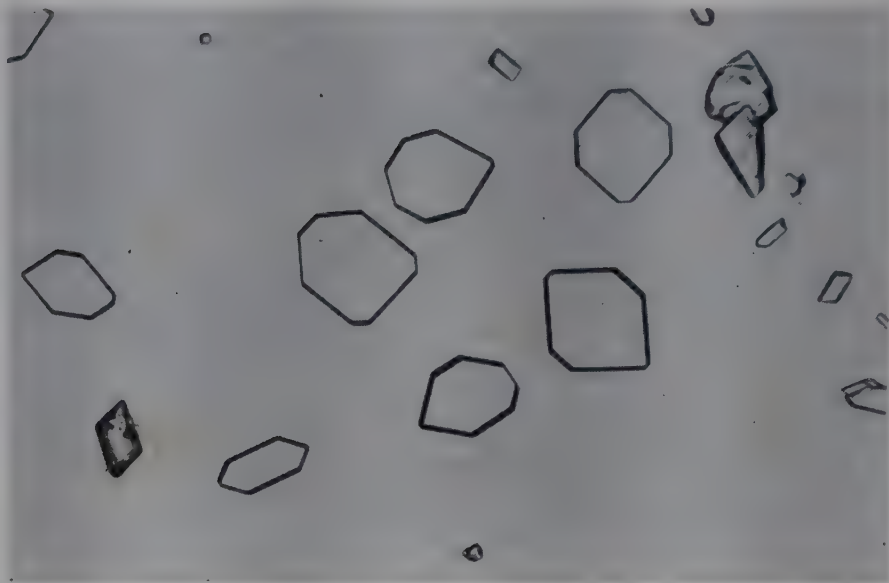


Fig. 46.—Sulfanilamide crystals. (Courtesy Merck & Co., Inc., Rahway, N. J.)

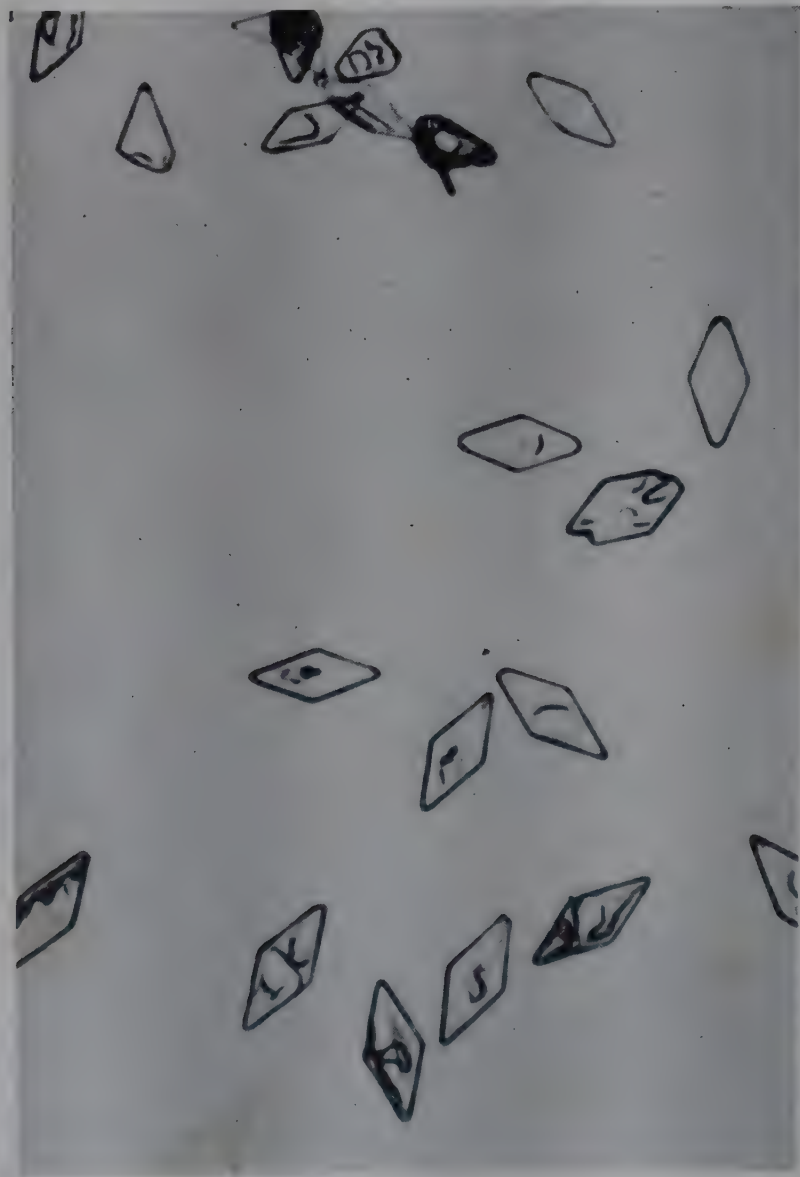


Fig. 47.—Sulfapyridine crystals. (Courtesy Merck & Co., Inc., Rahway, N. J.)

of patients taking these drugs may be due to damage to the renal filter apparatus. Lehr and his co-worker state that the colloids may assume a protective action by inhibiting the precipitation of poorly soluble substances. The increase in solubility by a colloid-chemical mechanism would render additional explanation to the fact that the concentration of sulfapyridine, sulfathiazole, sulfadiazine, and their acetyl derivatives in the urine of patients treated with these compounds can significantly exceed the solubility of the same drugs in water and normal urine. This fact may be important in preventing precipitation of concrement formation in the urinary tract.



Fig. 48.—Sulfathiazole crystals. (Courtesy Merck & Co., Inc., Rahway, N. J.)

Lehr and Antopol showed photomicrographs (Figs. 49 to 53) which indicate the specific nature of the crystals of each of these compounds. They did not claim, however, that these pictures show *all* forms possible nor did they deem it necessary to employ more photographs to show slight variations in the shapes described. They did state, however, that with the aid of these illustrations recognition of the particular drug has been possible on the first microscopic examination of urinary sediment in almost every case.

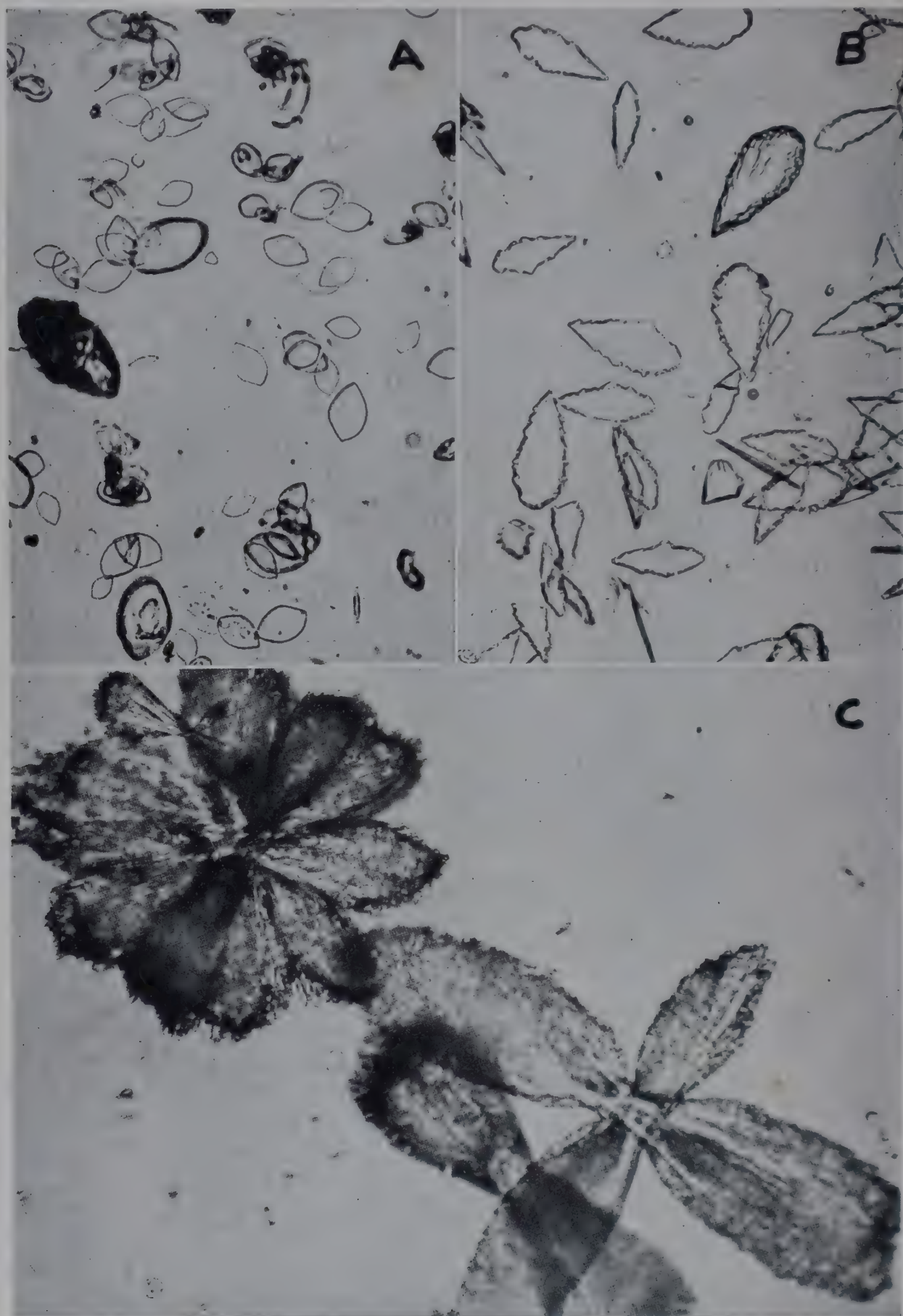


Fig 49.—Urinary crystals of acetylsulfapyridine. A, "Whetstones." Most common form; varying considerably in size (0.008 by 0.01 to 0.05 by 0.08 mm.), they are transparent, colorless, and usually show notching or serration of one or both edges. B, "Arrowheads" (0.016 by 0.56 to 0.04 by 0.92 mm.), similar to A, but wedge-shaped, presenting one sharp point and pronounced sawlike dentification of one or both sides. The base is rounded and shows irregular notchings. C, Centrally waisted sheaves composed of needlelike crystals, usually rather large, as also pictured by Sadusk and co-workers; (largest crystal in the photograph 1 by 4 mm.). These sheaves may conglomerate and form cross- and starlike structures. (Enlargement $\times 176$.) (Lehr, David, and Antopol, William: Specific Morphology of Crystals Appearing in the Urine During Administration of Sulfanilamide Derivatives, *Am. J. Clin. Path.* 12: 4, 1942.)

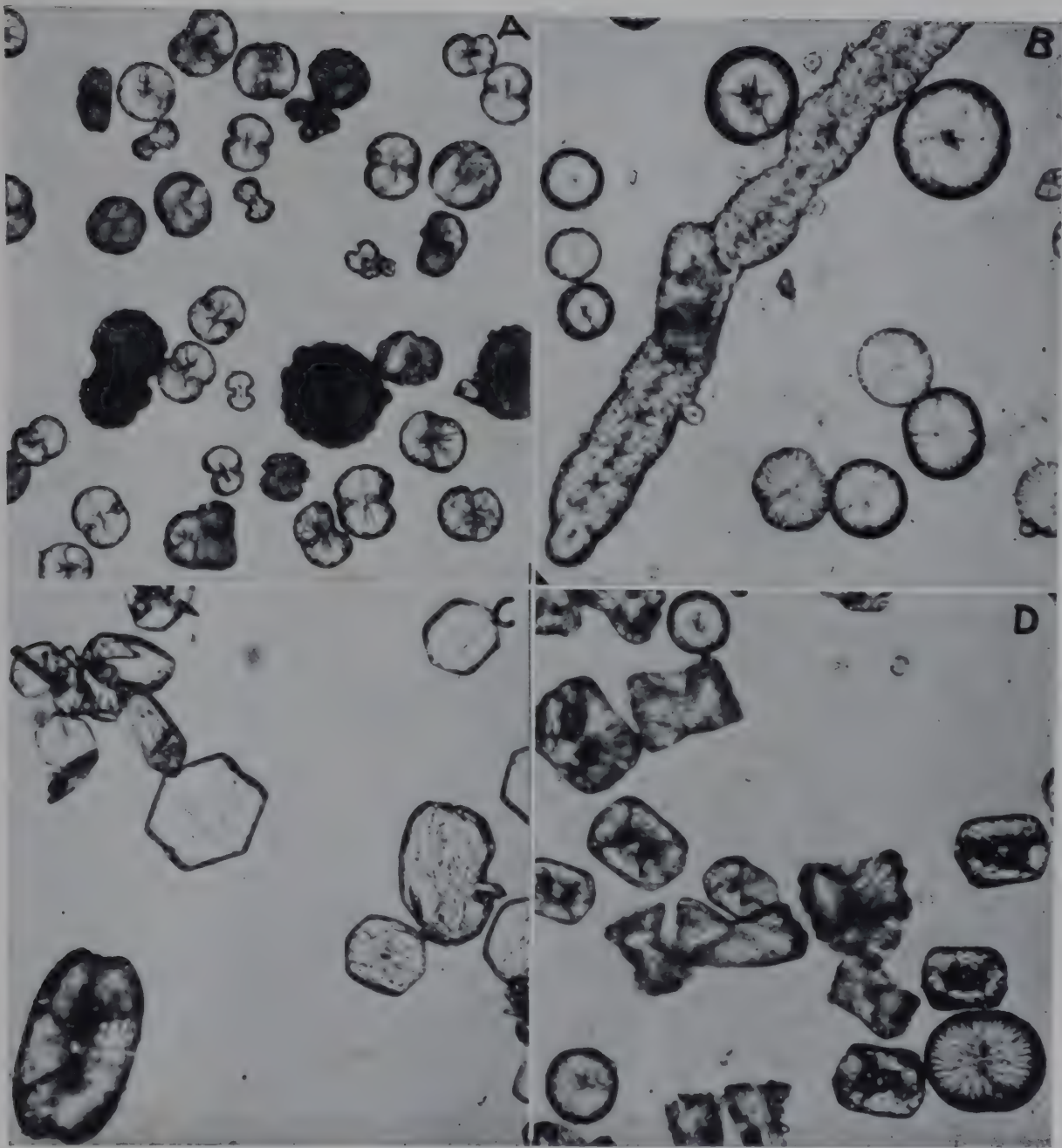


Fig. 50.—Urinary crystals of acetylsulfathiazole. *A*, *Dumb-bells* or “shocks of wheat with central binding”; the crystals start as small slender sheaves and grow in such a way that the two half-circle parts finally close up to form a rosette (0.004 by 0.06 to 0.06 by 0.08 mm.). *B*, *Rosettes* (0.04 to 0.1 mm.), the large rodlike structure, consisting of acetylsulfathiazole, may represent a “crystalline cast.” On microscopic examination both crystal forms (*A* and *B*) appear amber green and show pronounced radial striation. *C*, *Hexagonal Platelets* (0.04 by 0.06 to 0.15 by 0.2 mm.). Sometimes very thin glass-clear, more often thick amber green with a distinct envelope-like pattern. Some of the crystals may reach considerable size and two opposite edges may appear indented resulting in shapes as shown in the right lower corner of the photograph. *D*, Demonstrates the simultaneous presence in the same urinary specimen of the 3 forms outlined in *A*, *B* and *C*, a rather frequent observation. (Enlargement $\times 125$.) (Lehr, David, and Antopol, William: Specific Morphology of Crystals Appearing in the Urine During Administration of Sulfanilamide Derivatives, *Am. J. Clin. Path.* 12: 4, 1942.)

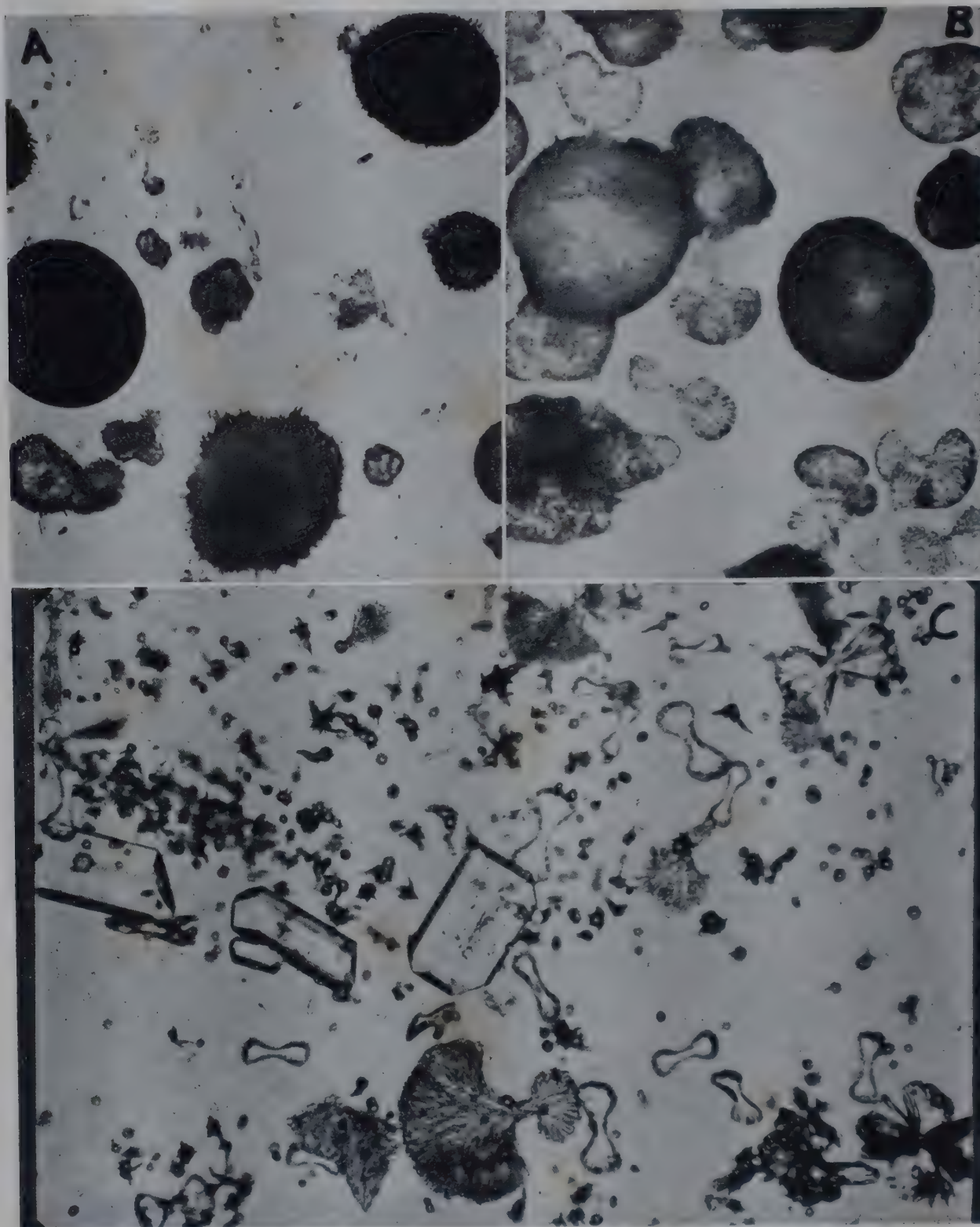


Fig. 51.—Urinary crystals of sulfadiazine and acetylsulfadiazine. *A*, Dark, muddy green globules, sometimes covered with needlelike processes (chestnut burr forms), consisting almost entirely of free sulfadiazine (0.03 to 0.16 mm.). *B*, Among "globules" containing free sulfadiazine, "shocks of wheat with excentric binding" and "shell" forms, consisting mainly of acetylsulfadiazine. Like the corresponding sulfathiazole crystals they are amber green and have a conspicuous radial striation (0.02 to 0.14 mm.). *C*, Urinary sediment of a patient who had been switched from sulfathiazole to sulfadiazine treatment 24 hours before voiding the urine specimen containing these crystals. A perfect "shock of wheat with excentric binding" (acetylsulfadiazine) can be seen among symmetrical dumb-bells (acetylsulfathiazole). (Also present are typical phosphate and monourate crystals. (Enlargement $\times 150$.) (Lehr, David, and Antopol, William: Specific Morphology of Crystals Appearing in the Urine During Administration of Sulfanilamide Derivatives, *Am. J. Clin. Path.* 12: 4, 1942.)

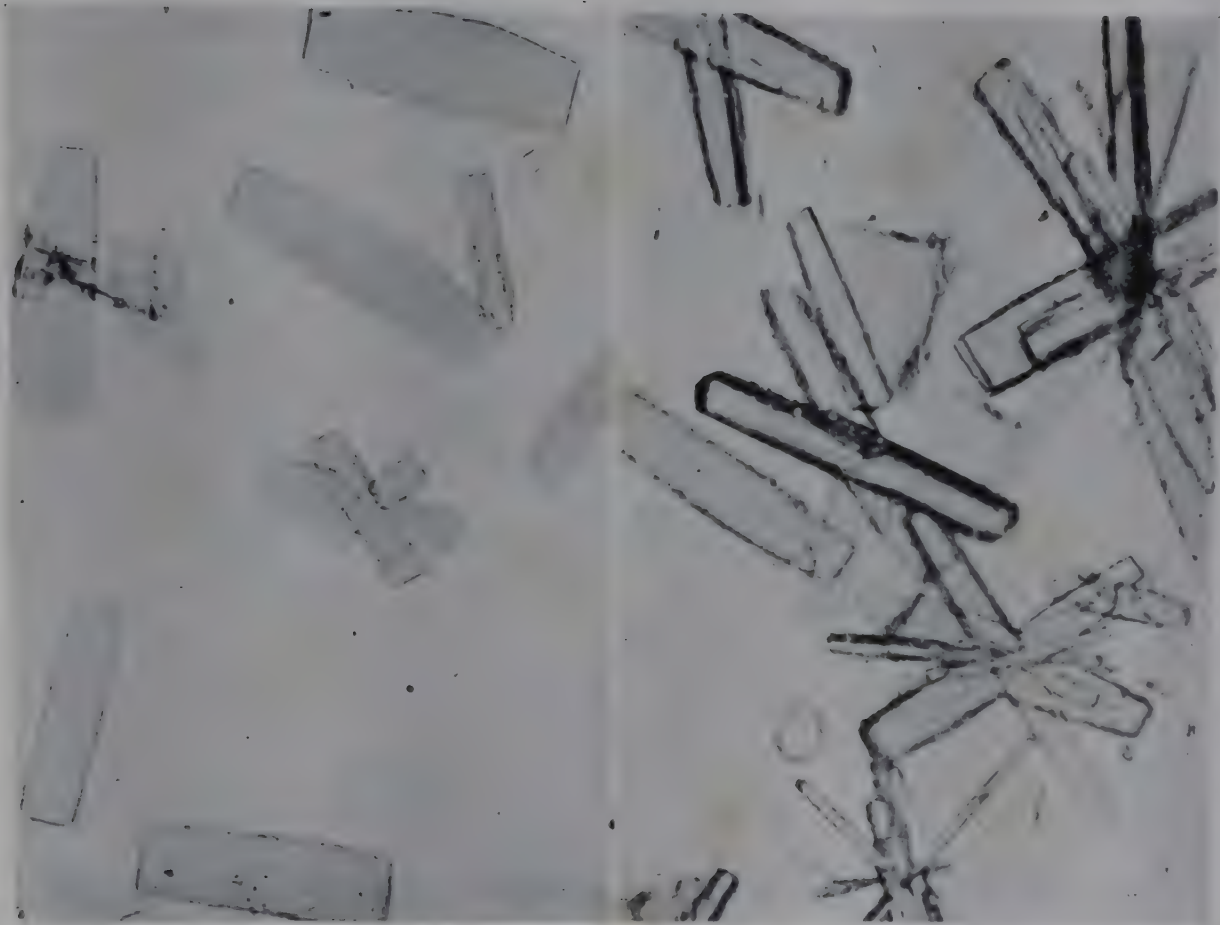


Fig. 52.—Urinary crystals of acetylsulfaguanidine. Simple rectangular oblong plates with slight bulging in the long axis. Markedly varying in size (0.02 by 0.09 to 0.05 by 0.24 mm.), these plates are either glass-clear or have a fine meshlike pattern, which makes them appear like isolated plant cells or slender tapeworm proglottides. They tend to conglomerate, forming cross- or starlike structures. Repeatedly slender sheaves of needle crystals were seen interspersed among the plates (center in the upper part of the right photograph). (Enlargement $\times 138$.) (Lehr, David, and Antopol, William: Specific Morphology of Crystals Appearing in the Urine During Administration of Sulfanilamide Derivatives, *Am. J. Clin. Path.* 12: 4, 1942.)



Fig. 53.—Featherlike tufts of needle crystals, amber green, observed in the urine of a patient receiving sulfamethylthiazole. Unfortunately these crystals were not saved for analysis. The general discontinuance of sulfamethylthiazole therapy makes further investigations difficult. (Enlargement $\times 250$.) (Lehr, David, and Antopol, William: Specific Morphology of Crystals Appearing in the Urine During Administration of Sulfanilamide Derivatives, *Am. J. Clin. Path.* 12: 4, 1942.)



Fig. 54.—The crystalline form of the sodium salt of penicillin G. (Courtesy Merck & Co., Inc., Rahway, N. J.)

UROLITHIASIS¹

Composition of Urinary Calculi

Despite a vast amount of clinical observation and experimental research the problem of urinary lithiasis remains unsolved. Causation is believed to be linked with nutrition, although many exceptions exist. The problem of prevention of recurrence in the susceptible individual has been studied at length, and results have not been too successful.

Methods of analysis of urinary calculi have been devised to ascertain the exact composition of urinary calculi with the end in view of utilizing this knowledge to prevent recurrence.

Optical and X-Ray Crystallography¹

Because of the inadequacies of chemical methods of analysis as applied to identification of urinary calculi, mineralogic methods have been applied, and these methods are presented here.

Urinary calculi can be divided into two general classes, crystalline and amorphous. The term "amorphous" has been loosely applied to phosphatic concretions which are grossly opaque, dull, and chalky, and apparently non-crystalline. With the exception of those exceedingly rare calculi which are

¹Prien, Edwin, and Frondel, Clifford: *J. Urol.* 57: 6, June, 1947.

composed of fibrin and bacteria it may be stated that all urinary calculi are crystalline. When the so-called "amorphous" calculi are broken up into a coarse powder and examined under a petrographic microscope, the fragments exhibit optical properties of crystalline substances, and also give distinct and characteristic x-ray diffraction patterns.

(a) **Optical Methods.**—Chemical compounds tend to form crystals when they pass from a liquid or gaseous state to the solid state. The external geometric form and internal molecular structure of such crystals are distinct, constant, and characteristic for any particular substance. Even when conditions in the environment are unfavorable for the development of visible geometric crystal faces, the formless crystalline mass has the same internal atomic structure.

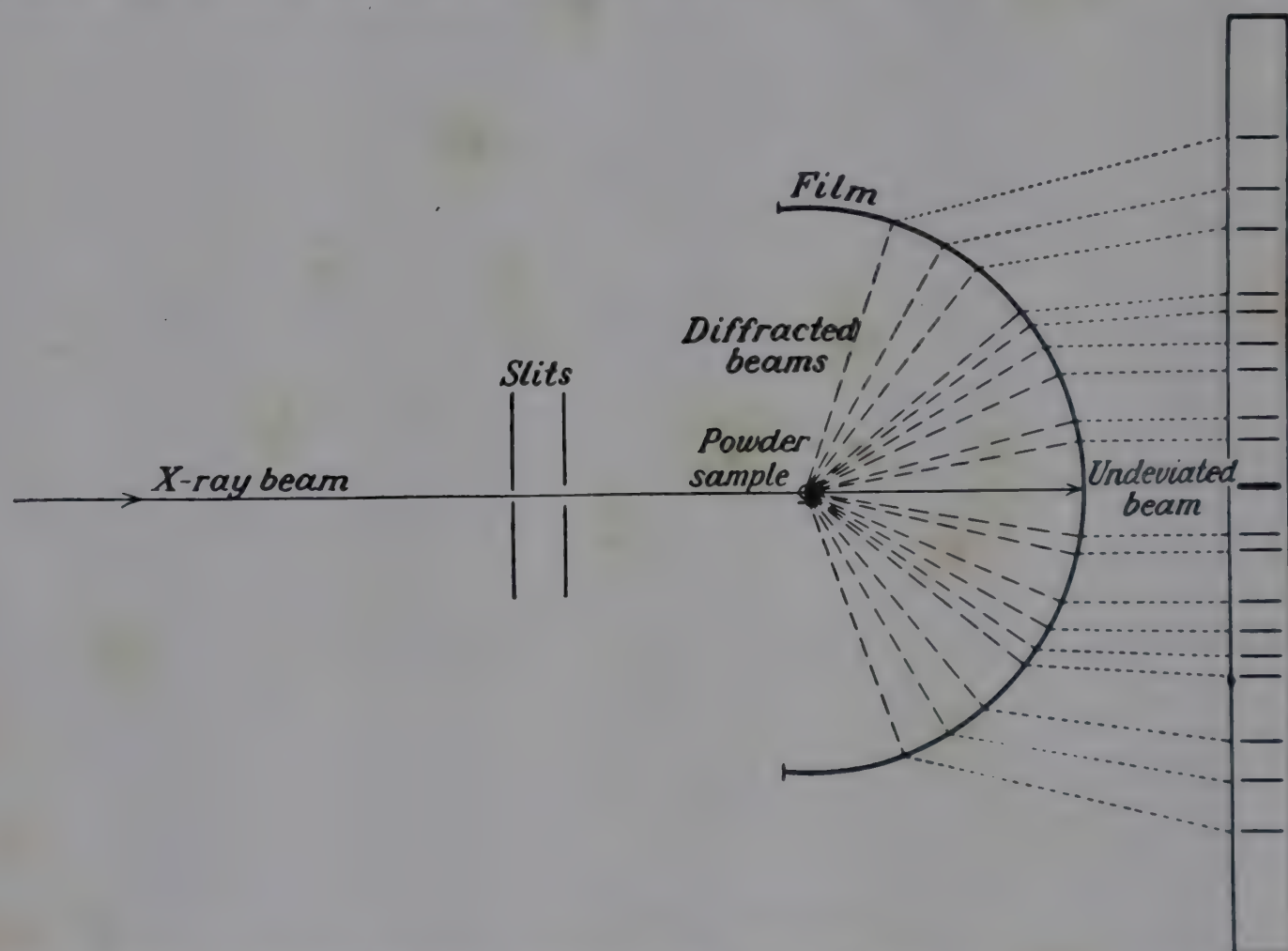


Fig. 55.—Diagram showing method of obtaining x-ray powder photographs. (From Prien and Frondel: *J. Urol.* 57: 949, June, 1947. By permission of the authors and publisher.)

Light passing through transparent crystalline material is influenced by the crystal structure according to the laws of optics, and these changes in transmitted light can be measured accurately. This results in data called "optical constants." The polarizing or petrographic microscope is a useful instrument for measuring external and internal physical properties of crystalline material. A series of compounds of known chemical composition is analyzed for optical constants and unknown substances are recognized by measuring their optical constants and comparing with prepared tables.

Another method is the so-called "immersion method" in which a minute quantity of coarsely ground calculus is placed upon a glass slide and immersed in a succession of liquids of known indices of refraction. A match is found for the indices of refraction. Other optical constants, including optical sign, axial

angle, dispersion, extinction angle, sign of elongation, etc., may be measured at the same time. A milligram of substance is usually required, but measurements can be made on a few microscopic grains by transferring them from slide to slide.

Constituents of urinary calculi are relatively few in number and markedly different in optical behavior, which makes the problem of identification simple. Only a few diagnostic optical tests need usually be applied to identify a substance. Confirmatory tests can be made at times.

(b) **Analysis by X-Ray Methods.**—(Method of Hull or of Debye and Scherrer)—the "Powder" method.

The method consists in irradiating a finely powdered sample of a substance with a beam of monochromatic x-rays and recording the diffracted (reflected) rays in a suitably arranged camera. The rays are reflected from planes of atoms in the substance, to be recorded as a series of lines upon a circular photographic film, forming a characteristic pattern for the substances irradiated. A diagram of the method is seen in Fig. 55. Two methods of identification may be used:

1. Spacings of the lines on the films are measured and the interplanar spacings are calculated for the substance in question. Comparison is made with a tabulated list of interplanar spacings of known chemical substances. This list is procurable from Dow Chemical Company or from the American Society for Testing Materials.

2. Comparison is made of the unknown patterns with standard x-ray patterns of known substances.

For this method of testing the size of the sample may be quite small, 1 mg., but 0.1 mg. or even less, will suffice. The sample may be first examined optically then ground to a fine powder and examined by x-ray. It is possible with this method to examine mixtures and to identify the several constituents and to estimate the approximate proportions of each.

Spacing data on x-ray powder diffraction may be obtained in the original article.¹

Technic of Optical Analysis

(1) **Examination With Ordinary Light.**—Instruments needed are a stereoscopic binocular dissecting microscope, lamp, biological dissection needles (a small machinist's vise fitted with a heavy needle is excellent), small mortar and pestle for powdering calculi, watch glasses to hold powdered samples, corked small glass vials or bottles, pair of cutting pliers or a dental extraction forceps for breaking large or firm stones.

Transfer samples of powder to the blade of a small knife. Use watch-maker's forceps or tweezers to transfer individual grains or crystals which have been spotted in a sample under magnification.

Fracture of the calculus permits inspection of such structural characteristics as texture, lamination, porosity, and the study of individual crystals, and is necessary if the nucleus is to be observed.

The calculus should be thoroughly dried before analysis is attempted.

1. Calculus is fractured.

2. External and fractured surfaces are inspected with the low magnification (10× or 20×) of a hand lens or binocular stereoscopic microscopic (dissecting microscope). Fig. 56.

¹Loc. cit., p. 117.

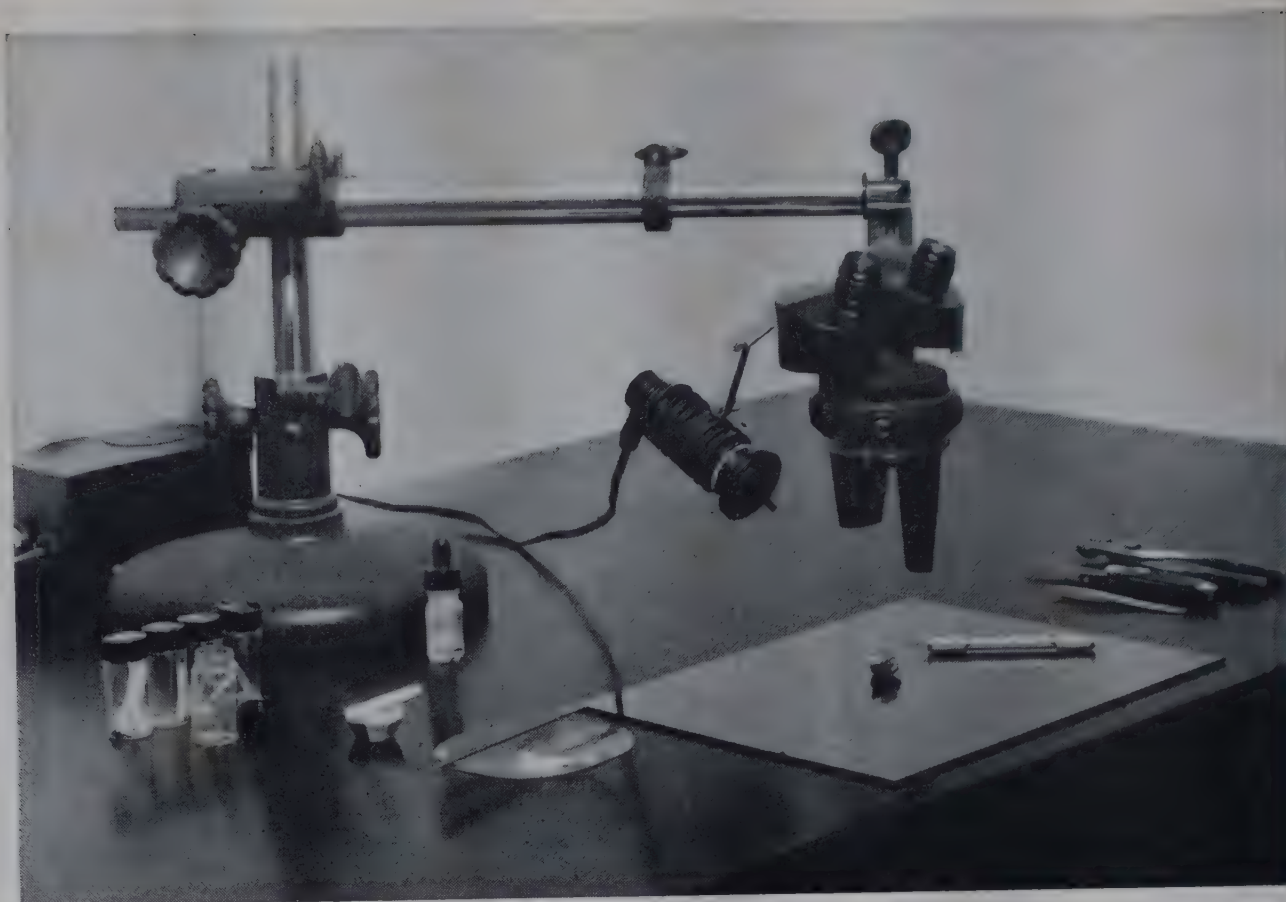


Fig. 56.—Equipment for examination of calculus with ordinary light. Includes binocular stereoscopic microscope, lamp (mounted on microscope here), pliers to fracture hard calculi, mortar and pestle to grind calculi, knife blade or spatula to transfer powder, watch glasses to hold powder, pointed tool for picking at laminations, nucleus, etc., stoppered bottles for filing small calculi and powder. (From Prien and Frondel: *J. Urol.* 57: 949, June, 1947. By permission of the authors and publisher.)

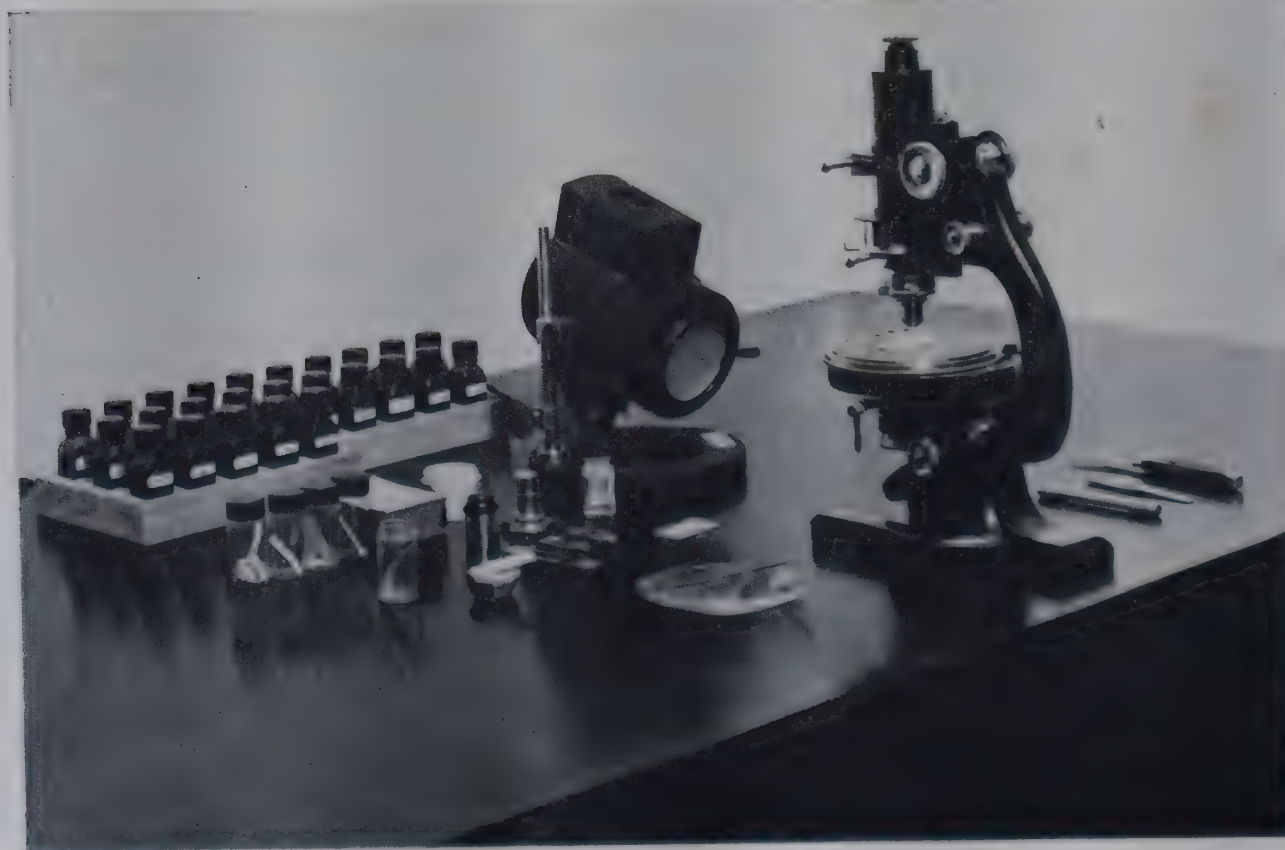


Fig. 57.—Equipment for examination of calculus with polarized light. Includes petrographic microscope, lamp, set of immersion liquids with graduated indices of refraction from 1.41 to 1.75 (in bottles in rack), glass slides and cover slips, also implements for examination with ordinary light. (From Prien and Frondel: *J. Urol.* 57: 949, June, 1947. By permission of the authors and publisher.)

(2) Examination With Polarized Light.—

1. Minute samples are removed with pointed tool and ground to a coarse powder in a mortar with pestle.

2. Powder is placed upon a glass slide, covered with liquid of known index of refraction, and a glass cover slip is placed over the specimen. Examine upon the stage of a polarizing microscope. Fig. 57.

Immersion liquids have indices from 1.41 to 1.75, graduated in steps of at least 0.01. Liquids of intermediate index, approximately accurate to 0.005 may be prepared by mixing equal droplets of liquids of two adjacent steps in the series. This may be done on the glass slide, but is not necessary in routine analysis. Accuracy to 0.01 is sufficient for the differentiation of all the substances in the accompanying determinative table.

To analyze calculi composed of several components it is possible to approximate the percentage of each component by powdering the entire calculus, stirring the fragments thoroughly to obtain a uniform mixture, and then making grain counts on random samples through the binocular and petrographic microscopes. It is possible to estimate the percentage composition to within 5 or 10 per cent, which is sufficient for most purposes.

Crystalline Components of Urinary Calculi

In his studies of urinary calculi, Prien et al. found that pure calcium oxalate calculi constituted 36.1% of the total; mixed calcium oxalate-apatite calculi comprised 31.0%; or 67.1% of the total were included in this group. These calculi usually, but not always, occur in acid sterile urine.

Pure magnesium ammonium phosphate hexahydrate, pure apatite, and mixed magnesium ammonium phosphate hexahydrate-apatite calculi comprised 19.5% of the total. These calculi usually, but not always, occur in alkaline infected urine.

Calcium hydrogen phosphate dihydrate occurred in 1.6% of the calculi.

Uric acid and cystine exist more frequently in pure than in mixed form and occurred in 6.1% and 3.8%, respectively, of the series.

Sodium acid urate occurred but once in the series and in microscopic amount. It was the only urate found.

Found in Present Study	Formula	Mineralogical Name
Calcium oxalate monohydrate	$\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$	Whewellite
Calcium oxalate dehydrate	$\text{CaC}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$	Weddellite
Magnesium ammonium phosphate hexahydrate	$\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$	Struvite
Carbonate-apatite	$\text{Ca}_{10}(\text{PO}_4)_6(\text{CO}_3)(\text{OH})_2$	Carbonate-apatite
Hydroxyl-apatite	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	Hydroxyl-apatite
Calcium hydrogen phosphate dihydrate	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	Brushite
Uric acid	$\text{C}_5\text{H}_4\text{N}_4\text{O}_3$	
Cystine	$\text{SCH}_2\text{CH}(\text{NH}_2)\text{—COOH}$	
Sodium acid urate	$\text{NaH} \cdot \text{C}_5\text{H}_2\text{O}_3\text{N}_4 \cdot \text{H}_2\text{O}$	
Not found but identity established		
Tricalcium phosphate	$\text{Ca}_3(\text{PO}_4)_2$	Whitlockite
Not found but identity acceptable		
Indigo	$\text{C}_{16}\text{H}_{10}\text{N}_2\text{O}_2$	
Xanthine	$\text{C}_5\text{H}_4\text{N}_4\text{O}_2$	

In a study of approximately 700 urinary calculi, only nine distinct crystalline substances were found. These are divided into groups: oxalates, phosphates, uric acid, urates, and cystine.

Calcium Oxalate

Calcium oxalate is the most frequent constituent found in urinary calculi.

Calcium Oxalate Monohydrate

Whewellite



This is more common than the dihydrate. The simplest form is the small smooth ovoid "hempseed" stone, usually multiple, and consists of concentric laminations, Fig. 58, 1 and 2.



Fig. 58.—Calcium oxalate monohydrate ($\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$) calculi. 1 and 2 are of the "hempseed" variety, small, smooth. 3 and 4 are of the "mulberry" variety, externally showing rounded mammillary surfaces. Scale in millimeters. (From Prien and Frondel: *J. Urol.* 57: 949, June, 1947. By permission of the authors and publisher.)

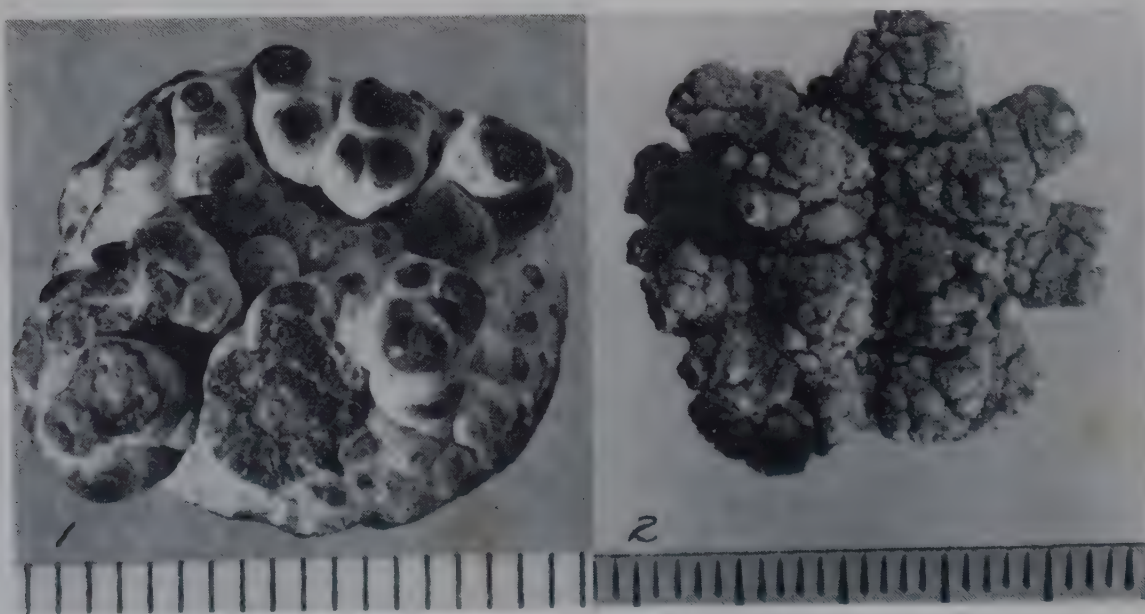


Fig. 59.—1, Calcium oxalate monohydrate calculus. Typical appearance of "mulberry" stone. Fracture of mammillary process shows concentric lamination and radial striation with central granular nucleus. 2, Calcium oxalate "jackstone," a central mass of calcium oxalate monohydrate with radiating blunt spinous processes. A fine deposit of calcium oxalate dihydrate crystals is precipitated over the exterior. Scale in millimeters. (From Prien and Frondel: *J. Urol.* 57: 949, June, 1947. By permission of the authors and publisher.)

The second type is the "mulberry" stone of variable size and shape with irregular rounded mammillary processes, Fig. 58, 3 and 4. On cross section these rounded protuberances are seen to be composed of radially striated aggregates. These radiating fibers are much finer than those of another fibrous constituent of calculi, $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, and may be almost microscopic, Fig. 59, 1. Some predominately fibrous (radially bladed) calculi may have a finely or coarsely granular nucleus of the same material, Fig. 59, 1.

The third type of calculus is the uncommon "jackstone" which is usually found in the bladder and consists of a dense central mass with radiating spicules, Fig. 59, 2.

Calculi composed of calcium oxalate monohydrate are occasionally porous or cavernous, especially toward the interior; or a particular layer in a laminated stone may be porous. The granular type of structure occurs most commonly in those calculi of mixed composition. Both the fibrous and granular calculi may show growth banding (lamination) marked by color differences parallel to the surface of the stone. Calcium oxalate monohydrate usually forms calculi which are extremely dense, tough, and hard, and is usually the substance found when it is reported that a calculus resisted crushing or sawing.

External surface—usually smooth and may have a lustrous or varnished appearance.

External color—light honey brown to red brown or black brown, or occasionally blue to greenish blue.

Fractured surface—usually some shade of brown.

Finely granulated calculi—relatively light colored.

Nuclei of other substances, uric acid and apatite, are sometimes observed and calcium oxalate monohydrate may occur as nuclei in uric acid and other stones. Calcium oxalate dihydrate is the most common associate of calcium oxalate monohydrate and usually occurs as well-formed crystals attached to or embedded in the surface of the monohydrate stone. Apatite also is a common associate; magnesium ammonium phosphate hexahydrate (struvite), calcium hydrogen phosphate dihydrate (brushite), and uric acid are infrequent.

Calcium oxalate monohydrate is monoclinic in crystallization and possesses cleavages on (001), (010), and (110). Cleavages can be recognized in relatively large single crystals but are not easily seen in fibrous or fine-granular aggregates. Hardness— $2\frac{1}{2}$ to 3. Specific gravity—2.23. Optical properties:

Indices	
X = 1.491 = b	Biaxial positive (+)
Y = 1.555	2V = 84°. Dispersion $r < v$, weak.
Z = 1.650	Extinction angle $Z \wedge c = 31^\circ$.

It is easily distinguished optically from calcium oxalate dihydrate, $\text{CaC}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$ by its high birefringence and high Z index of refraction.

Occasionally the granular type of calculus may show well-defined tiny crystals upon its surface, Fig. 60, 1, 2, 3.

Calcium Oxalate Dihydrate

Weddellite



These generally occur as relatively well-formed crystals, shaped as in Fig. 60, 4, which range in size up to 2 mm. on an edge. They are identical with the familiar octahedral or "envelope" crystals seen in ordinary urinary sediments. The crystals are usually attached to the body of the stone by their edges. Calculi composed of pure calcium oxalate dihydrate consist either of loosely intergrown aggregates of individual crystals, Fig. 60, 1, or of compact, interlocking aggregates with a coarsely crystallized surface, Fig. 60, 2. A secondary crop of smaller crystals may be super-deposited. In calculi of mixed internal composition a

granular structure may exist with no development of crystals. No nucleus is found in most of the loosely aggregated calculi. Pure dihydrate calculi are much less common than pure monohydrate stones.

Color—pale yellowish white to honey brown; lighter than the monohydrate. The two hydrates are frequently associated; the dihydrate usually occurs as a surface incrustation of well-formed crystals upon or slightly embedded in the monohydrate. The contrast between the two forms may be quite striking, Fig. 60, 4. The dihydrate is usually of later formation than the monohydrate, but in part has overlapped the closing stages of growth of the monohydrate. Much less commonly the two may be intimately associated in those granular oxalate calculi which lack any regularity of structure. Apatite is less commonly associated with the dihydrate. Frequently the two oxalates and apatite exist together. Infrequent associates are with calcium hydrogen phosphate dihydrate (brushite), cystine, and uric acid.

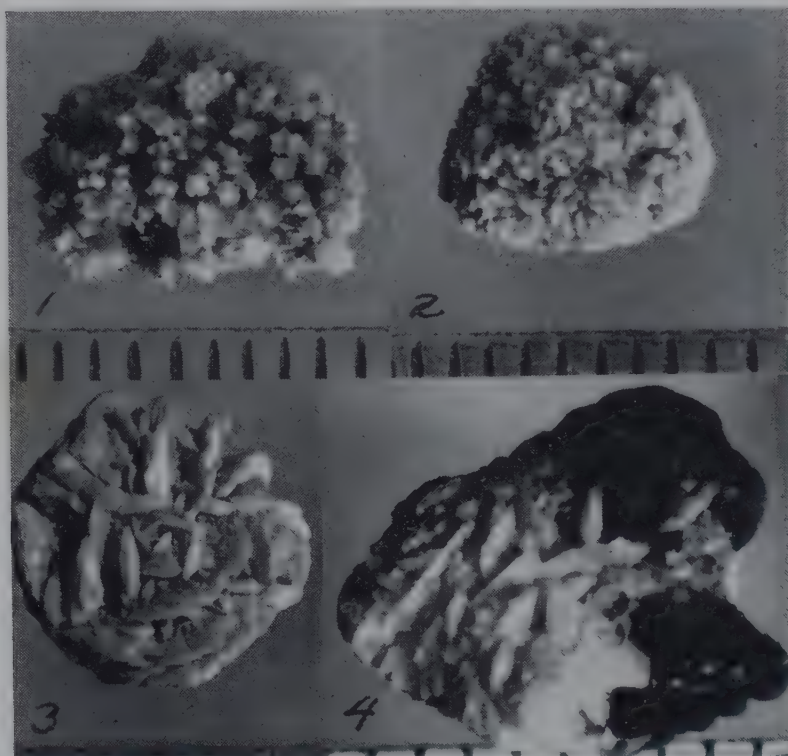


Fig. 60.—Calcium oxalate dihydrate ($\text{CaC}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$) calculi. 1, Loose aggregate of crystals. 2, Compact aggregate of crystals. 3, Unusually large crystals seen edgewise. 4, Dihydrate crystals (white) deposited upon calcium oxalate monohydrate calculus (black). Knifelike edges of dihydrate crystals are well shown. Scale in millimeters. (From Prien and Frondel: *J. Urol.* 57: 949, June, 1947. By permission of the authors and publisher.)

Prien believes that both the monohydrate and the dihydrate are direct products of crystallization from the urine.

Calcium oxalate dihydrate is tetragonal in crystallization. It has no cleavage. Hardness—4. Specific gravity—1.99. Optical properties:

Indices

O = 1.523

Uniaxial positive (+)

E = 1.544

It is distinguished optically from calcium oxalate monohydrate by its much lower birefringence and lower indices of refraction. Microscopically the crystals commonly show growth banding (lamination) parallel to the crystal faces.

Optical data are lacking for the trihydrate. Oxalates other than the dihydrate and the monohydrate have not been observed in urinary calculi by

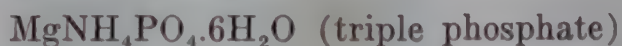
Prien. Prien has found stone-forming patients who produce pure monohydrate or dihydrate calculi as well as some who produce concretions composed of a mixture of the two hydrates. The urines of patients who produce dihydrate calculi may contain fused masses (gravel) of the familiar "envelope" crystals. It appears that calculi may at times be formed by the normal crystalline salts in urine, contrary to statements in the literature. The significance of the two phases of calcium oxalate in calculi is being investigated.

Phosphates

Phosphates in urinary calculi present a complex problem which has not previously been resolved by chemical methods of study. Four different phosphates have been found. Two of these, magnesium ammonium phosphate hexahydrate (struvite) and apatite, form the great bulk of the material. A third, calcium hydrogen phosphate dihydrate (brushite), is relatively uncommon, being found but 12 times in 700 calculi. The fourth, tricalcium phosphate (whitlockite), has been reported in several instances. Magnesium ammonium phosphate, "triple phosphate," is usually identified in the chemical analysis of a phosphatic stone. Commonly reported with it are "calcium phosphate" and "calcium carbonate"; these separate substances have not been found in this study, but are combined in one complex compound, carbonate-apatite, a carbonated calcium phosphate.

Magnesium Ammonium Phosphate Hexahydrate

Struvite



This is usually associated with apatite in calculi. Pure calculi composed of it are quite uncommon, producing a densely granular creamy-white appearance somewhat resembling lump sugar, Fig. 61, 1. A suggestion of columnar structure is frequently present. In association with apatite, this substance forms the so-called "staghorn" calculi which occur in alkaline infected urine.

Color, dirty to creamy white; structure, typically cellular or columnar, Fig. 62, often resembling cancellous bone.

Triple phosphate and apatite crystals exist together in all degrees of admixture. A study showed that approximately half were composed predominately of the one substance and half were composed of the other.

Well-formed single crystals of magnesium ammonium phosphate hexahydrate may be observed in open cracks in banded or laminated calculi of apatite and also projecting into cavities in porous stones. Crystals may also be found embedded in the central portions of some calculi where the apatite may be loose, fluffy, and grossly structureless. They also occur in urinary sediments as the well-known "knife-rest" or "coffin-lid" crystals. Crystals of typical shapes are shown in Fig. 70, 6, 7, 8. They lack a center of symmetry in that the upper and lower halves are not mirror images.

Calcium oxalate monohydrate is an uncommon associate of magnesium ammonium phosphate hexahydrate and the dihydrate is rare. One calculus was observed to have a nucleus of calcium oxalate monohydrate, an intermediate layer of calcium hydrogen phosphate dihydrate, and a periphery of magnesium ammonium phosphate hexahydrate. Uric acid was also observed as the nucleus of a calculus composed of magnesium ammonium phosphate hexahydrate and apatite.

It is orthorhombic-hemimorphic in crystallization. Crystals have a nearly perfect cleavage on (001) and an indistinct cleavage on (010).

Hardness—2; specific gravity—1.71. Optical properties:

$$X = 1.495 = a$$

$$Y = 1.496 = c$$

$$Z = 1.504 = b$$

Biaxial positive (+)

$2V = 37^\circ$. Dispersion $r < v$, strong.

Microscopically it is easily distinguished by its very low birefringence and low indices of refraction.

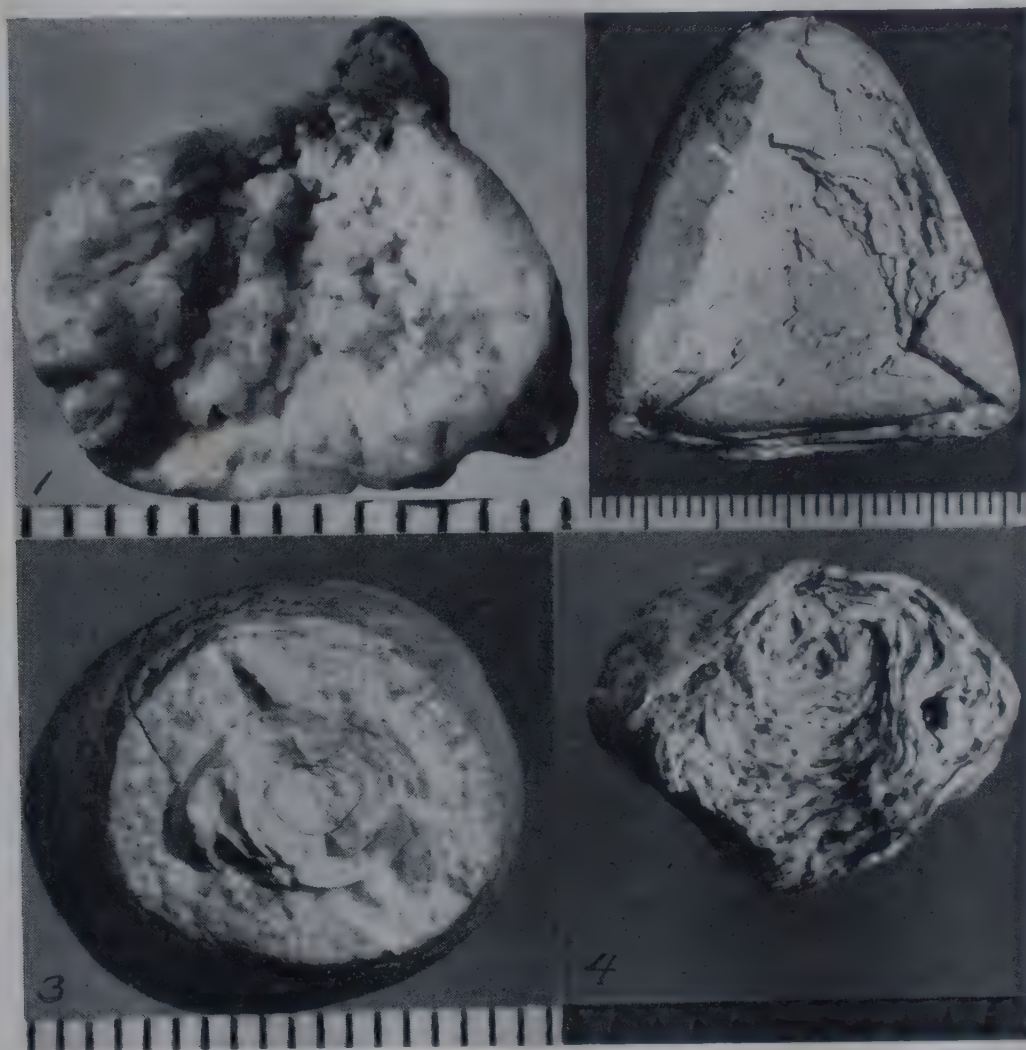


Fig. 61.—Phosphatic calculi. 1, Practically pure $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$, "triple phosphate," showing developing columnar structure. 2, 3, and 4, Pure apatite calculi. 2, Shows flaking of laminations from dry calculus (triangular outline due to molding by bladder, four similar stones being present). 3, Three types of apatite structure are shown, central loose fluffy nucleus, intermediate zone of very dense laminations, peripheral zone of less dense laminations. 4, The alternating light and dark laminations are all pure apatite. Scale in millimeters. (From Prien and Frondel: *J. Urol.* 57: 949, June, 1947. By permission of the authors and publisher.)

Apatite

Carbonate-apatite

Hydroxyl-apatite



The term "apatite" refers to a group of compounds which have an analogous chemical composition and identical crystal structure. They are mostly minerals. Carbonate-apatite and hydroxyl-apatite are complex phosphates occurring in calculi and belong to this group.

Calcium hydrogen phosphate dihydrate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) uncommonly exists in calculi and tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) occurs rarely. Prien has not found calcium carbonate (CaCO_3) in apatite calculi.

Carbonate-apatite and hydroxyl-apatite can be identified as apatite only by optical or x-ray methods. They are then differentiated from each other by releasing carbon dioxide gas from carbonate-apatite. If a large amount of gas is evolved from an apatite substance occurring in calculi it is inferred to be wholly or preponderantly carbonate-apatite; if little or no gas is evolved it seems to be wholly or preponderantly hydroxyl-apatite. They should be considered collectively and called "urinary apatite" to distinguish them from mineral apatite which usually contains chlorine or fluorine ions.

Apatite commonly occurs with magnesium ammonium phosphate hexahydrate as one of the major components of phosphatic calculi. It is, by far, the most common phosphate present in calculi. Pure apatite calculi are not common; they are fine-grained, soft, and compact, usually with concentric lamination, both on macroscopic and microscopic scale, Fig. 61, 2, 3, 4. Successive laminae may vary somewhat in color and coherency, and some laminae may be relatively hard with a translucent glassy appearance. Inner portions of some stones are loose and powdery, Fig. 62.

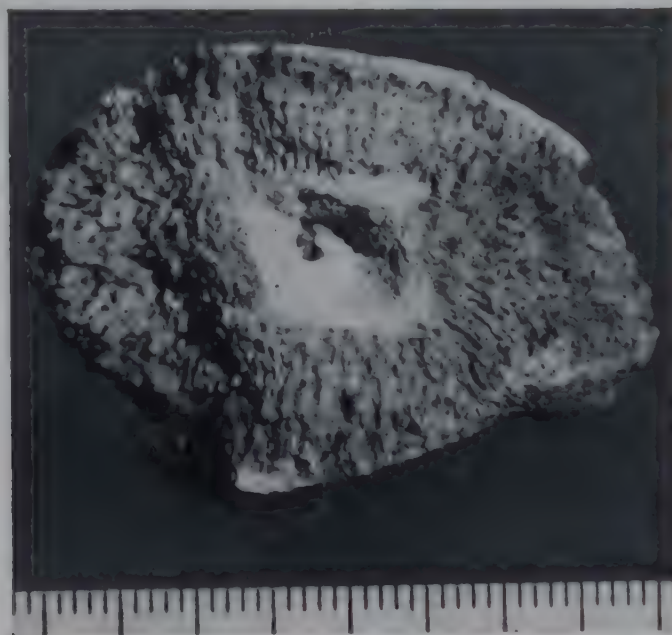


Fig. 62.—Fractured surface of "staghorn" calculus. The central nucleus of apatite is surrounded by the columnar structure of $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$, "triple phosphate." The intercolumnar spaces are filled with apatite. Scale in millimeters. (From Prien and Frondel: *J. Urol.* 57: 949, June, 1947. By permission of the authors and publisher.)

Color—chalky white, cream white, yellow brown, sometimes brown. Pure apatite—alternating lamination of white and brown, Fig. 61, 4, in which the brown layers represent a condensation of the substance. Brown layers may be translucent or even transparent.

In mixtures with magnesium ammonium phosphate hexahydrate in "staghorn" calculi the apatite is softer and may usually be rubbed to a fine powder with the finger. In porous granular calculi composed of the oxalates it exists in interstices or cavities in variable amounts. Commonly the interspaces in the loose aggregates of calcium oxalate dihydrate crystals may be partially or completely filled with the substance; Fig. 63 shows such aggregates. It has also been observed with calcium hydrogen phosphate dihydrate, cystine, and uric acid. Calculi of apatite and magnesium ammonium phosphate hexahydrate commonly occur in alkaline infected urines.

It is hexagonal in crystallization; well-formed crystals being found only in nature. Hardness—5; specific gravity—2.95 to 3.10. Natural crystals are optically negative, with *O* and *E* indices in the range from 1.61 to 1.64, depending on the kind and amount of isomorphous substitution. In urinary calculi, it appears as extremely fine-grained masses, never as visible crystals and is isotropic. This character is due to the submicroscopic size of the crystals composing the mass with resultant aggregate polarization. Index of refraction—1.55 to 1.61, most samples falling between 1.55 and 1.59. This variable and low index is partly due to a large content of adsorbed and capillary water. Index may vary between different parts of the same sample. Some grains may show weak double refraction. Some grains ordinarily are colorless, but may be pale yellow, brown, reddish brown, or greenish yellow due to foreign pigmenting matter.

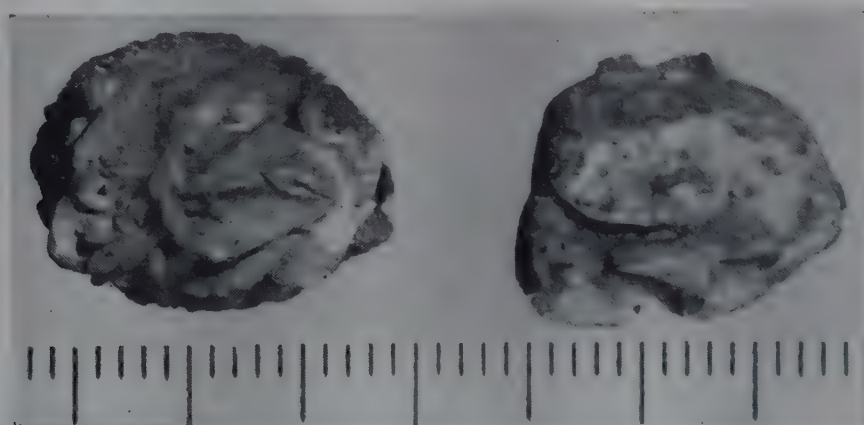


Fig. 63.—Apatite upon calcium oxalate dihydrate. The linear edges of calcium oxalate crystals are seen upon the left, projecting through the apatite. On the right the calcium oxalate has been almost completely "submerged" by apatite, only the tips of the crystals being visible as irregularities in the surface. Scale in millimeters. (From Prien and Frondel: *J. Urol.* 57: 949, June, 1947. By permission of the authors and publisher.)

It differs from other components of calculi in its isotropic or nearly isotropic character and the absence of crystal outlines. It may be confused with colloidal organic material when seen through the petrographic microscope because both are isotropic. The colloid frequently appears as a coating on crystals from which it may be differentiated readily. It is very confusing if on apatite which does not form crystals. Test for CO_2 which may be liberated from carbonate-apatite.

Place a few coarse grains on a glass slide and cover with a cover glass.

Run a drop of dilute hydrochloric acid under the edge of the cover glass.

If carbonate-apatite is present a brisk effervescence ensues which may be watched through low magnification.

To confirm that this is CO_2 add a drop of 10% barium hydroxide under the cover glass. A white cloud of barium carbonate forms about the stone, showing presence of CO_2 . Failure to produce effervescence in material having the constants of carbonate-apatite indicates the presence of hydroxyl-apatite.

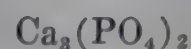
Colloidal apatite calculus is undoubtedly ordinary carbonate-apatite. " $\text{Ca}_3(\text{PO}_4)_2$ " prostatic calculi of Huggins and Bear appear to be composed of carbonate-apatite. The powder pattern is actually that of hydroxyl-apatite. While the percentage difference in composition between the two substances is very little, their atomic structures are quite unlike.

Carbonate-apatite is the principal constituent of calcifications variously formed in the lacrimal duct, prostate gland, tuberculous lymph nodes, appendix, testes, and walls of the bronchi as a consequence of senile degeneration and disease. It also occurs as a minor constituent in arteriosclerotic plaques.

A calcium phosphate kidney stone that afforded small amounts of CO_2 and H_2S when dissolved in acid was found to be carbonate-apatite containing a small amount of divalent sulphur in isomorphous substitution, and may be termed a sulphidic apatite.

Tricalcium Phosphate

Whitlockite



Whitlockite is rhombohedral in crystallization. No cleavage. Hardness—5; specific gravity—3.2; indices: $O = 1.629$, $E = 1.626$; uniaxial negative.

Prien has identified whitlockite in a testicular calcification. It was creamy white, very fine-grained, and almost porcellanous in appearance, and contained a small amount of CO_2 . It appeared isotropic, due to the very small particle size of the aggregate, and had a variable index of 1.59 to 1.60. Some of the natural material has also been found to contain CO_2 in isomorphous substitution analogous to carbonate-apatite. Urinary whitlockite probably has the same general appearance and optical properties as apatite. Probably the " $\text{Ca}_3(\text{PO}_4)_2$ " reported in older descriptions of urinary calculi was apatite in most instances.

Calcium Hydrogen Phosphate Dihydrate

Brushite



This is an uncommon phosphate occurring in urinary calculi, having been found but 12 times in 700 calculi. In at least 3 instances the calculi were present as bladder stones accompanying benign hypertrophy of the prostate and with urinary infection. Because it is an acid phosphate and is usually associated with the oxalates in calculi it is reasonable to suppose that it forms in an acid urine. It was accompanied several times by carbonate apatite, which also occurs commonly with the oxalates. In the single instance in which it occurred with magnesium ammonium phosphate hexahydrate the latter was present as a peripheral layer which could be explained as the result of a change in the reaction of the urine to the alkaline side subsequent to the precipitation of the calcium hydrogen phosphate dihydrate.

It may exist as a layer overlying a nucleus of calcium oxalate monohydrate, as bladed crystals in granular calcium oxalate monohydrate or apatite, or (one calculus) it may approach a pure state (low per cent of apatite). Figs. 65; 64, 4; 64, 1.

In pure form it has a radially fibrous or bladed structure, Fig. 64, 2, 3. A crudely divergent tufted or fernlike structure may sometimes be seen in cross section, Fig. 64, 2. The ends of these tufts produce a uniformly nodular appearance, Fig. 64, 1.

Color—creamy white or yellowish white, occasionally light brown.

Structure—moderately soft. Fracture surfaces tend to have a somewhat pearly or silky luster, due to perfect cleavage. It differs from fibrous calcium oxalate monohydrate in this characteristic and in that the latter is harder, darker, and lacks a good cleavage along the length of its fibers.

They are monoclinic in crystallization, with perfect cleavages on both (010) and (301). Hardness—2 to 2½. Specific gravity 2.32. Optical properties:

Indices	Biaxial positive (+)
X = 1.539	2V = 87 degrees
Y = 1.545	Dispersion perceptible v < r.
Z = 1.551 = b	Extinction Y ∧ a = 18°.

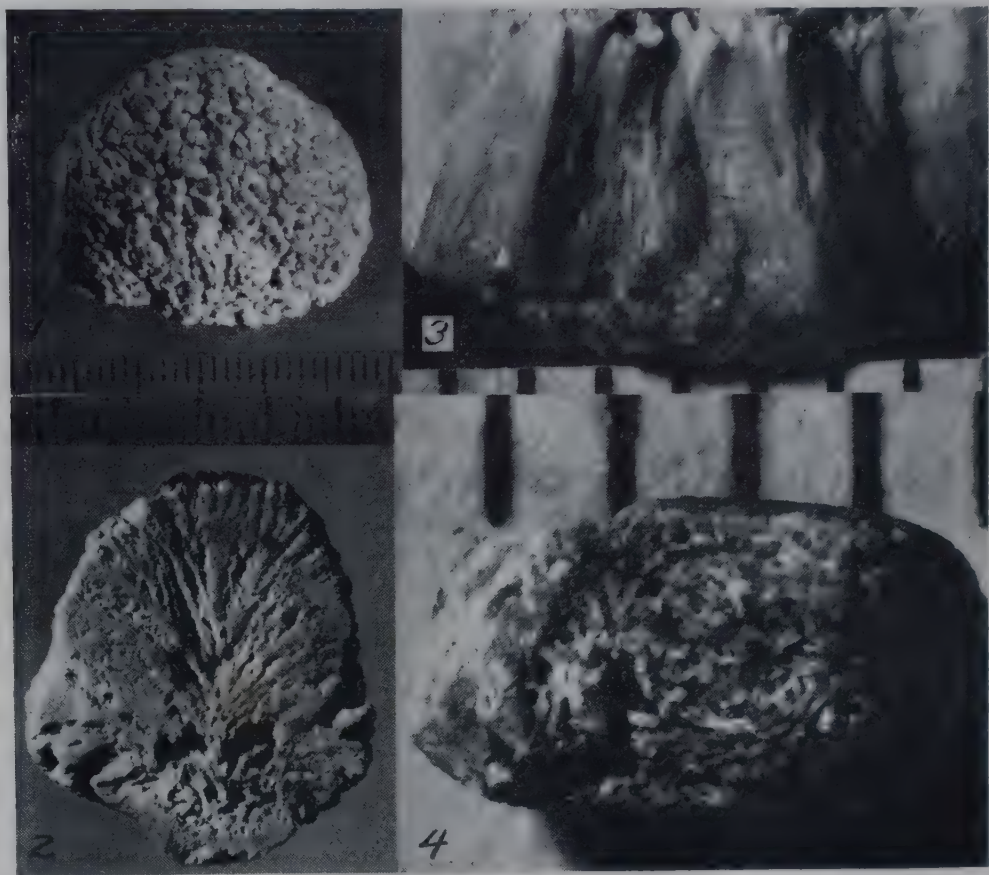


Fig. 64.—Calcium hydrogen phosphate dihydrate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) in calculi. 1, External surface of rare pure $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ calculus, showing ends of tufts of crystals, producing nodular structure. 2, Cross section of same calculus showing divergent tufted structure. 3, "Palisade" formation of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, due to cleavage along fibers which are well shown. 4, Bladed crystals of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ in a granular calcium oxalate monohydrate calculus. Scale in millimeters. (From Prien and Frondel: J. Urol. 57: 949, June, 1947. By permission of the authors and publisher.)

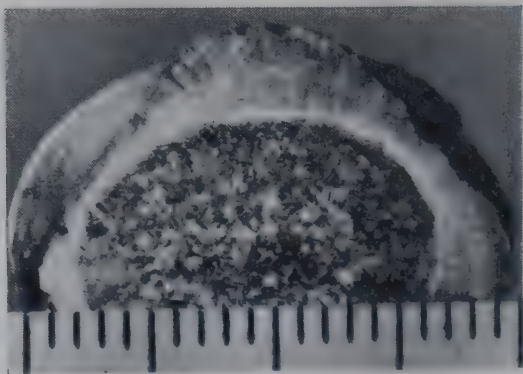


Fig. 65.—Multilayered calculus. Nucleus of granular calcium oxalate monohydrate, second layer (almost invisible) of calcium oxalate dihydrate, third layer (broad) of laminated calcium hydrogen phosphate dihydrate, peripheral layer (very thin) of calcium oxalate dihydrate. Scale in millimeters. (From Prien and Frondel: J. Urol. 57: 949, June, 1947. By permission of the authors and publisher.)

Microscopically, its low birefringence is approximated by magnesium ammonium phosphate hexahydrate and calcium oxalate dihydrate from which it is easily distinguished by its higher indices and bladed crystals.

Uric Acid



In calculi uric acid occurs most often in a pure state. Its most common associates in calculi of mixed composition are apatite and calcium oxalate monohydrate. It occurs sometimes as the nucleus in calculi composed of calcium oxalate monohydrate or of apatite. Apatite may also be found in the interstices of coarsely porous uric acid calculi. Calcium oxalate monohydrate has also been found as the nucleus of a uric acid stone.

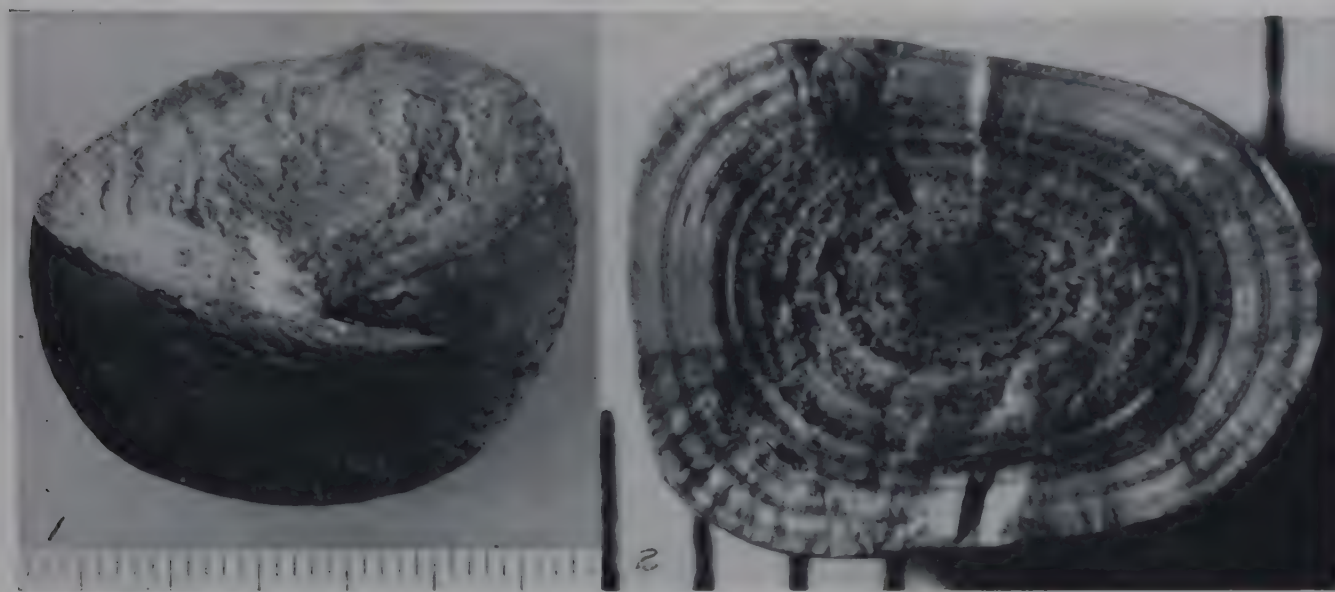


Fig. 66.—Uric acid calculi. 1, Dense fine-grained structure shown on fractured surface. 2, Alternating light and dark laminations are all pure granular uric acid. Central nucleus of uric acid crystals. Scale in millimeters. (From Prien and Frondel: *J. Urol.* 57: 949, June, 1947. By permission of the authors and publisher.)

They have dense fine-grained structure and are relatively heavy, Fig. 66, 1; oblate or flattened pebble-like shape with smooth but not polished surface is typical; warty surfaces may be observed. Cross sections usually show a concentric lamination of variable coarseness with radial striation, Fig. 66, 2. Alternating light and dark laminations are of identical composition, the dark layers probably being due to pigmentation. Some calculi have a granular appearance without evidence of lamination. Nucleus is usually composed of un-oriented uric acid crystals. Color—some shade of brown with which red or orange may be mixed.

It is orthorhombic in crystallization and has good cleavage. Hardness— $2\frac{1}{2}$; specific gravity 1.89. Some crystals are rectangular laths or rounded lozenge-shaped tablets flattened on (001), Fig. 70, 9, 10. Optically, it is bi-axial. Optical sign is unknown. Optic plane is parallel to (001), with $X = 1.573$, $Y = ?$, $Z = 1.830$. Polymorphous or hydrated form, stable at relatively low temperature, is said to have $X = 1.53$ and $Z = 1.73$. Pure recrystallized uric acid is white to yellowish white and pale brownish white. It has a marked tendency to adsorb foreign pigmenting substances; the pigmented material usually is pleochroic under the microscope, with absorption $Z > X$. The so-called “brick-dust” precipitate from urine is pigmented uric acid.

Microscopically, the high birefringence of uric acid is approached only by calcium oxalate monohydrate among the common stone-forming substances. It

is distinguished from the latter by finding grains which have indices of refraction above 1.65 (the highest index of calcium oxalate monohydrate), and failure to find grains with indices below 1.573 (the lowest index of uric acid).

Urates

Many urates, including both the acid and normal salts of NH_4 , Na, K, and Mg have been described in urinary calculi. Identification is questionable or inadequate. Urates occur less commonly in calculi than would appear from the results of chemical analysis alone. Sodium acid urate has been found but once and in microscopic amount in a series of approximately 700 urinary calculi. It

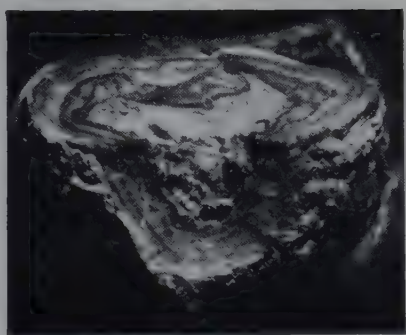


Fig. 67.



Fig. 68.

Fig. 67.—Sodium acid urate in fractured uric acid calculus. Narrow dark zones extending around calculus are composed of needlelike crystals of sodium acid urate which extend across zone. Diameter of calculus 0.4 cm. (From Prien and Frondel: *J. Urol.* 57: 949, June, 1947. By permission of the authors and publisher.)

Fig. 68.—High-power view of sodium acid urate crystals seen upon fractured surface of calculus in Fig. 67 ($\times 30$). (From Prien and Frondel: *J. Urol.* 57: 949, June, 1947. By permission of the authors and publisher.)

has been found by Prien in several tiny plaques attached to the apices of renal papillae and in the so-called uric acid infarcts of renal papillae. In the single occurrence in a calculus a large central nucleus of uric acid was surrounded by a dark gray-brown zone with a radially fibrous structure; a peripheral layer of apatite was present, Fig. 67. Dissection showed the soft fine silky needles of sodium acid urate easily visible under magnification, Fig. 68.

Sodium acid urate ($\text{NaC}_5\text{H}_3\text{O}_3\text{N}_4\cdot\text{H}_2\text{O}$) is monoclinic or triclinic in crystallization. Crystals are lath or needle-shaped and possess two perfect cleavages and probably a third imperfect cleavage. Material is very soft and plastic.

Color—white, yellowish-white, or cream yellow. Crystal aggregates have a silky luster. Microscopically crushed fragments appear as rhomboidal plates or

laths bounded by cleavage faces with extinction parallel to one of the cleavages. Optically positive (+) with $X = 1.448$, $Y \sim 1.75$, $Z > 1.84$. Interior acute angle of the rhomboidal plates is approximately 65° , as shown in Fig. 71.

Ammonium acid urate was found in a bladder stone from a bulldog.

Another substance was found once in microscopic amount in a depression upon the surface of a calcium monohydrate calculus. It remains unidentified but was probably a urate.

Cystine



Cystine usually occurs in the pure state in calculi but sometimes is found admixed with small amounts of apatite, and, rarely, calcium oxalate monohydrate. Occasionally it may exist in a pure state as the nucleus of a calculus otherwise composed of the above constituents.



Fig. 69.—Cystine calculi composed of aggregates of hexagonal crystals. Soft pearly luster, difficult to photograph. Diameter of calculi 2-3 mm. (From Prien and Frondel: *J. Urol.* 57: 949, June, 1947. By permission of the authors and publisher.)

Cystine stones commonly consist of porous aggregates of relatively well-formed short hexagonal prisms or hexagonal tablets, Fig. 69. Well-crystallized stones sometimes have a compact granular center. The color is honey-yellow to yellowish white. The luster is waxy. The calculi are very soft unless they are densely granular. An occasional cystine calculus may be so fine-grained and dense as to resemble uric acid externally.

Cystine is hexagonal in crystallization and possesses a perfect cleavage on (0001). Hardness—2. Specific gravity—2.06. Optical properties:

Indices	
O = 1.700	Uniaxial negative (—)
E = 1.640	

Cystine crystals commonly have the form of hexagonal tablets, as shown in Fig. 70, 5. The substance is easily recognized under the microscope, affording grains with a hexagonal outline which yield a centered uniaxial negative interference figure, with $O = 1.700$. The crystals and cleavage fragments usually show internal growth bands parallel to the external hexagonal crystal faces.

Other Substances

Prien failed to find xanthine and indigo in his study. Because of their extreme rarity it seems reasonable to believe that these substances do occur in

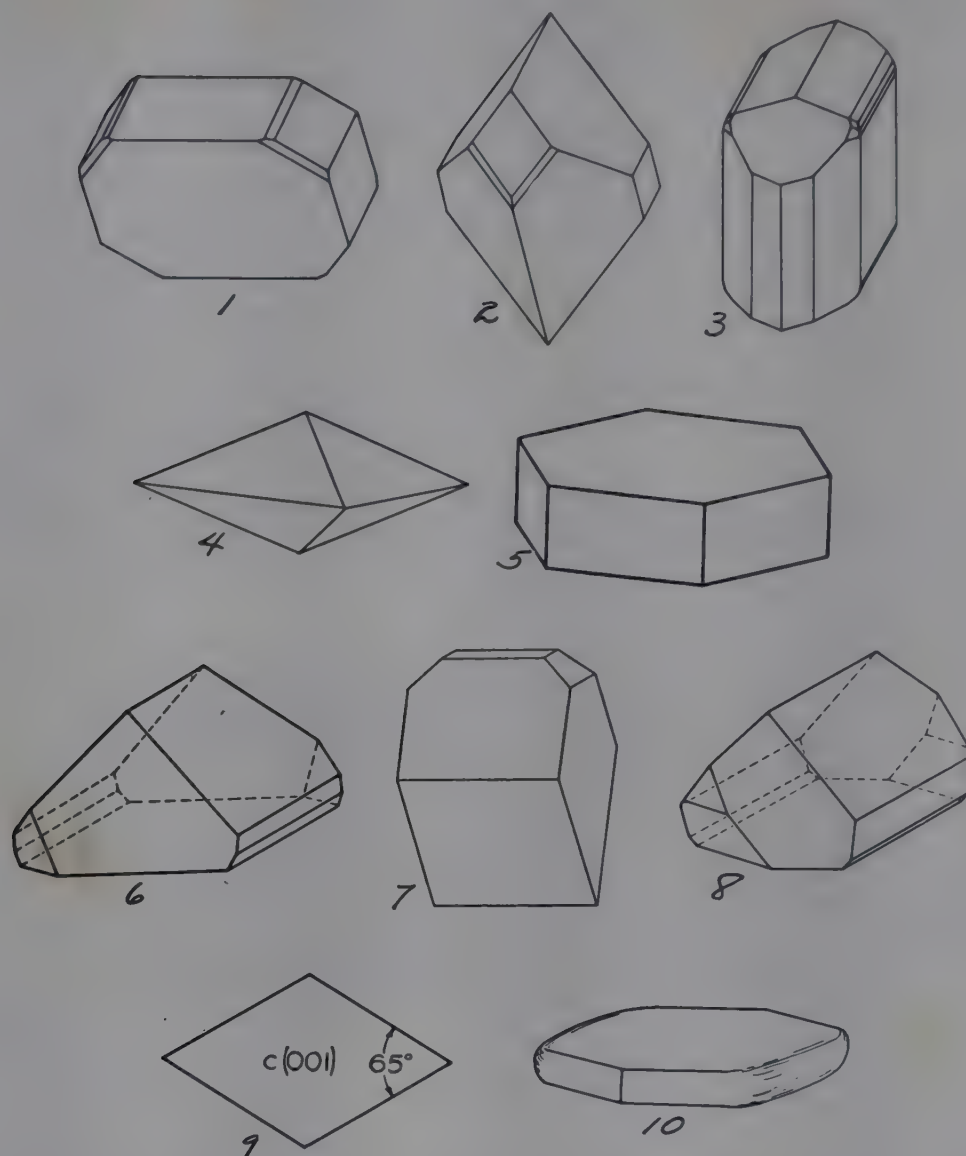


Fig. 70.—Forms of crystals seen upon the surfaces of calculi. 1, 2, and 3, Calcium oxalate monohydrate, uncommon, actual size 0.03-0.1 mm. 4, Calcium oxalate dihydrate, common, actual size up to 2.0 mm. 5, Cystine, common, actual size 0.1-0.2 mm. 6, 7, 8, Magnesium ammonium phosphate hexahydrate, common, actual size 0.5-1.0 mm. 9, 10, Uric acid, uncommon, actual size 0.2 mm. (From Prien and Frondel: J. Urol. 57: 949, June, 1947. By permission of the authors and publisher.)

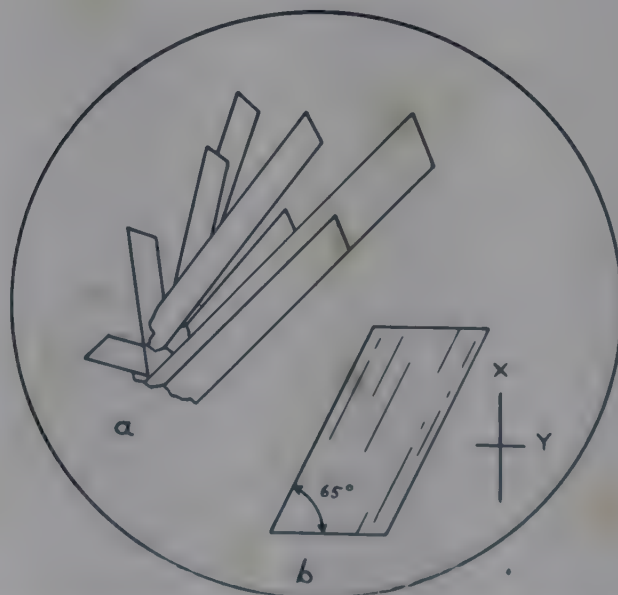


Fig. 71.—Sodium acid urate crystals ($\times 315$). (From Prien and Frondel: J. Urol. 57: 949, June, 1947. By permission of the authors and publisher.)

calculi. It seems reasonable to predict that small amounts of other substances may be detected in calculi by physical methods. Cholesterol has been reported. Prien has found cholesterol crystals in urine and in gallstones but not in calculi.

Calcium Carbonate

Aragonite	CaCO ₃
Calcite	
Vaterite-B or μ -calcite	

Calcium carbonate is commonly reported to occur in phosphatic calculi. Prien et al. have not encountered it. Development of effervescence when a calcareous stone is treated with dilute acid is not necessary evidence for the occurrence of calcium carbonate; in all instances examined by Prien it was due to the presence of carbonate-apatite.

Calcium carbonate crystallizes in three distinct forms and may be said to be polymorphous. The phenomenon is due to the geometrically different manner in which the constituent atoms are arranged in the crystal structure of the three substances. As a consequence the different forms may vary markedly in physical and chemical characters. A common example is provided by diamond and graphite, both of which are pure carbon. Calcium carbonate occurs as aragonite, calcite, and vaterite-B or μ -calcite. All three phases have been recognized as constituents of gallstones. So far as is known no specific phase has been identified in human urinary calculi. Calcium carbonate concretions are said to occur in herbivora.

Distinction between the three calcium carbonate phases may be effected optically if the grain size is sufficiently large, or in any case, by x-ray powder patterns. Aragonite, and possibly calcite as well, would probably appear as fine-grained or fibrous aggregates.

Aragonite.—

Aragonite is orthorhombic in crystallization with a distinct cleavage on (010) and indistinct cleavages on (110) and (011). Hardness— $3\frac{1}{2}$ to 4. Specific gravity—2.95. Optical properties:

X = 1.530 = c	Biaxial negative (—)
Y = 1.680 = a	2V = 18 degrees
Z = 1.685 = b	Dispersion r > v, weak.

Calcite.—

Calcite is rhombohedral in crystallization and has a good rhombohedral cleavage on (1011). Hardness—3. Specific gravity—2.715. Optical properties: uniaxial negative with O = 1.658 and E = 1.486. The E' index afforded by grains resting on cleavage surfaces is 1.566.

Vaterite-B or μ -Calcite.—

Vaterite-B is hexagonal in crystallization and occurs in artificial preparations as tiny hexagonal or spherulitic aggregates. Optical properties: uniaxial positive with O = 1.550 and E = 1.640.

TABLE 7
DETERMINATIVE TABLE OF OPTICAL CONSTANTS OF URINARY CALCULI

NAME	CRYSTAL SYSTEM	SIGN	X OR O	Y	Z OR E	2V	DISPER- SION	EXTINCTION ANGLE	DISTINGUISHING FEATURES
Calcium oxalate monohydrate Whewellite	Monoclinic	+	1.491 = <i>b</i>	1.555	1.650	84°	$r < v$ weak	$Z \wedge c = 31^\circ$	Distinguished from uric acid by indices; no index above 1.650; has index below 1.573
Calcium oxalate dihydrate Weddellite	Tetragonal	+	1.523		1.544				
Magnesium NH_4PO_4 hexahydrate Struvite	Orthorhombic	+	1.495 = <i>a</i>	1.496 = <i>c</i>	1.504 = <i>b</i>	37°		$r < v$ strong	
Carbonate-apatite	Hexagonal	Isotropic	Index variable, usually 1.55-1.59 (extreme range 1.520-1.610)						Isotropic; spherulitic; effervesces with HCl
Hydroxyl-apatite	Hexagonal	Isotropic	Index variable, as above						Isotropic; spherulitic; no effervescence with HCl
Calcium hydrogen PO_4 dihydrate Brushite	Monoclinic	+	1.539	1.545	1.551 = <i>b</i>	87°	$v < r$ perceptible	$Y \wedge a = 18^\circ$	Coarse radial fibers
Uric acid	Orthorhombic	?	1.573	?	1.830			Parallel to cleavage	Yellow color; see under ca. ox. mono.
Sodium acid urate	Monoclinic or triclinic		1.448	1.75	1.84			Parallel to cleavage	Rare; needlelike crystals with internal acute angle = 65°
Cystine	Hexagonal	-	1.700		1.640				Hexagonal crystals; good cleavage
Tricalcium phosphate Whitlockite	Hexagonal	-	1.629		1.626				Rare; like apatite

Calcium Sulphate Dihydrate**Gypsum**

Calcium sulphate has been said to occur occasionally as a urinary deposit but has not been observed in calculi by Prien et al. Gypsum, the particular phase which would be stable as a urinary deposit, is monoclinic in crystallization. Hardness—2; specific gravity—2.32. Gypsum has a perfect cleavage on (010) and imperfect cleavages on (111) and (100). Optically, gypsum is biaxial positive, with $X = 1.521$, $Y = 1.523$, $Z = 1.530$, and $2Z = 58^\circ$. The optic plane is parallel (010), the direction of perfect cleavage. Strongly inclined dispersion, with $r > v$.

Calcium Acid Phosphate**Monetite**

Monetite has been reported as a urinary sediment in carnivorous animals but has not been reported from human beings. Crystals are rhomboidal plates of laths and are triclinic in crystallization. Optically, monetite is biaxial positive (+), with moderately large $2V$, and $X = 1.600$; $Y = 1.614$; $Z = 1.631$. In comparison to brushite, $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, monetite forms artificially at relatively higher temperatures from acid solutions.

Cholesterol

Cholesterol has been described in urinary calculi; it occurs rarely as a heavy suspension of crystals in urine and in hydrocele fluid. It crystallizes in thin, transparent rhomboidal plates of varying size, often imbricated and united to one another. A rhomboidal notch frequently exists at one corner. The crystals should be recognizable at sight. Melting point 147.5° to 148.5° . Perfect cleavage (010). Optical properties: monoclinic in crystallization. $Z = 1.566$; $Y = 1.532$; $X = 1.520$. $2V = 60$ degrees \pm .

Tribasic Magnesium Phosphate Hydrate**Bobierrite**

Magnesium phosphate has been said to occur rarely as a urinary deposit but has not been observed by Prien et al. The phase stable as a urinary deposit probably is the octahydrate, bobierrite. This substance is monoclinic in crystallization, with a hardness of 2 and a specific gravity of 2.2. Optically biaxial positive, with $X = 1.510$, $Y = 1.520$, $Z = 1.543$. $2V = 71^\circ$, weak dispersion, with $r < v$. Extinction angle Z to $c = +29^\circ$. Optic plane perpendicular to (010).

Chemical Analysis of Urinary Calculi

In examining the larger urinary calculi chemically, the most valuable data are obtained by examining each of the concentric layers separately. Saw the calculus through the nucleus and separate the various layers. Material may also be obtained by scraping enough powder from each layer to carry out the examination. If the latter is adopted, the layers should not be separated.

Murexide Test

To a small amount of unknown material in a small evaporating dish, add 2 to 3 drops of concentrated nitric acid. Evaporate to dryness over a water-bath. If uric acid is

present, a red or yellow residue remains which turns purplish red after cooling the dish and adding a drop of very dilute ammonium hydroxide. The color is due to the formation of ammonium purpurate, or murexide. If potassium hydroxide is used instead of ammonium a purplish violet color due to the production of the potassium salt is obtained. The color disappears upon warming; with certain related bodies (purine bases) the color persists under these conditions.

Table 8 shows a scheme proposed by Heller for the chemical examination of urinary calculi and will be found very useful in determining their composition. Reduce the calculus to powder and proceed as directed.

Kamlet's Method

Table 8 is designed to give an outline of the pure chemical analysis of calculi. A very useful technic has been introduced by Kamlet.¹ In this method, as in the one just described, the larger calculi are cut up, and each of the concentric layers is subjected to a separate analysis. Small calculi are examined in toto. If possible, 100 mg. of powdered material are examined.

1. Describe the calculus as to shape, size, color, weight, and appearance on transection.
2. The specimen for analysis is divided into two equal parts. One portion is reserved for the detection of cystine, xanthine, urostealith, fibrin, cholesterol, and indigo. The other portion is macerated to a fine powder in a clean, dry porcelain mortar and added to 50 c.c. of 10 per cent hydrochloric acid (sp. gr. 1.05) in a small Pyrex flask. Effervescence at this point indicates the presence of carbonate ion. The mixture is gently boiled for five minutes, filtered through ashless filter paper and the filtrate is evenly distributed into five clean test tubes 6 inches by 1 inch.
3. One tube is rendered faintly alkaline with 10 per cent sodium hydroxide solution, and 5 c.c. of Nessler's reagent is added. A brown turbidity or sediment (the iodide of Millon's base) indicates the presence of ammonium ion.
4. One tube is rendered faintly alkaline with 10 per cent sodium hydroxide solution and then cautiously reacidified with dilute acetic acid. One cubic centimeter of arsenophosphotungstic acid and 4 c.c. of 5 per cent sodium cyanide solution is added. A blue color developing within sixty seconds indicates uric acid or urate ion.
5. One tube is rendered faintly alkaline with 10 per cent sodium hydroxide solution and then reacidified with dilute acetic acid. A white turbidity or precipitate indicates the presence of calcium oxalate. If no turbidity results after standing for ten minutes, 5 c.c. of a saturated ammonium oxalate solution is added. A white precipitate or turbidity at this point indicates the original presence of calcium ion only (probably as calcium carbonate).
6. One tube is rendered ammoniacal with strong ammonia water, and 1 c.c. of a 0.5 per cent solution of p-nitrobenzeneazoresorcinol in 95 per cent alcohol is added. A blue color developing within thirty seconds indicates the presence of magnesium ion.
7. To the last tube is added 2 c.c. of acid molybdate reagent (equal parts of 10 N sulphuric acid and 7.5 per cent sodium molybdate solution) and 1 c.c. of dilute tin chloride (SnCl_2) solution (1 c.c. of a solution of 10 gm. of SnCl_2 in 25 c.c. concentrated hydrochloric acid diluted with 200 c.c. of water). A blue color developing within sixty seconds indicates the presence of phosphate ion.
8. The residual half of the specimen for analysis is agitated with 10 c.c. of chloroform. An indigo calculus will turn the chloroform blue. The chloroform is then decanted from the insoluble sediment.
9. A few drops of the chloroform extract are evaporated on a glass slide, and the residuum is stained with Sudan III. Residual fat globules on the slide indicate a urostealith calculus.
10. To the remainder of the chloroform solution is added 5 c.c. of acetic anhydride and 0.5 c.c. of concentrated sulphuric acid. A Liebermann-Buchard reaction (play of colors ending in a naphthol green shade) indicates the presence of cholesterol.

¹Kamlet, J.: *J. Lab. & Clin. Med.* 23: 321, 1937.

ON HEATING THE POWDER ON PLATINUM FOIL, IT

*Scheme proposed by Heller. (Hawk and Bergelm, ed. 6, p. 700.) P. Blakiston's Son & Co.

11. The insoluble sediment from Step 8 is divided into three parts. One part is dissolved in nitric acid and cautiously dried over a free flame. A yellow residue which turns orange on the addition of 10 per cent sodium hydroxide solution and deep red on warming, indicates the presence of xanthine.

12. To the second part of the insoluble sediment from Step 8 add 2 c.c. of Millon's reagent* and heat. A positive protein reaction (red precipitate) indicates the presence of fibrin in the calculus.

13. To the last part of the insoluble sediment from Step 8 add 2 c.c. of concentrated ammonia water, and agitate for two or three minutes. One drop of the resultant solution is allowed to evaporate on a slide. If cystine is present, six-sided platelets will separate on the spontaneous evaporation of the ammonia. For a confirmatory test, dissolve 5 to 10 mg. of powdered calculus in 5 c.c. of N/10 hydrochloric acid, add 2 c.c. of 5 per cent sodium cyanide solution, mix, and let stand ten minutes. Add 1 c.c. of a 0.5 per cent aqueous solution of 1:2-naphthoquinone-4-sodium sulphonate and 5 c.c. of a 10 per cent solution of sodium sulphite (Na_2SO_3) in N/2 sodium hydroxide solution. Mix and let stand for thirty minutes. In the presence of cystine a reddish brown color forms and will turn deep red on the addition of 1 c.c. of a 2 per cent solution of sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_4$) in N/2 sodium hydroxide solution.

SPECIAL URINE CHEMISTRY

For information regarding colorimeters, colorimetric technic, and other special chemical methods, see Chapter III, Blood Chemistry.

Determination of Urinary Diastase (Amylase) Somogyi

The procedure for the determination of urinary diastase (amylase) is essentially the same as in the case of blood serum or plasma (see pages 374 ff.). It is important, however, to adjust the pH of the urine to from 6.8 to 7.2. In the case of acid urines, add sodium carbonate; to alkaline urines, add acid phosphate. Phenol red may be used as an indicator. If dilution is necessary, use 0.5% sodium chloride. Somogyi states that unpublished results obtained in the laboratory of the Jewish Hospital, St. Louis, show that the diastase value of human urine is always the multiple of that of the blood serum, a changed ratio indicating impaired kidney function. (See page 374.)

Amylase in Urine (Diastase)

Method of Lium after Wholgemuth

Reagents.—

Substrate.—

Make a substrate to produce a 0.1% starch solution with a reaction of pH 6.7.

Sodium chloride	- - - - -	4.5 gm.
Disodium phosphate	- - - - -	4.11 gm.
Potassium acid phosphate	- - - - -	5.13 gm.
Soluble starch	- - - - -	1.0 gm.
Distilled water	- - - - -	1000.0 c.c.

Boil for half an hour to dissolve the soluble starch.

Filter and place in a stoppered flask.

If kept in a refrigerator, this reagent does not deteriorate for at least four weeks.

N/200 Iodine Solution.—

Dissolve 1 gm. of iodine
and 2 gm. of potassium iodide
in 1500 c.c. of distilled water.

*See p. 48 for formula.

Technic.—

Use the morning urine specimen.

Place 2 c.c. of the starch solution in each of a series of 10 tubes.

Dilute the urine 1:30 by adding 1 c.c. to 29 c.c. of distilled water.

Add to the series of tubes containing the starch 10, 9, 8, 7, 6, 5, 4, 3, 2, and 1 drop of diluted urine, respectively.

Take care that no saliva enters the pipette.

Shake and place in a water bath at 38° C. for 30 minutes.

Place in cold water to stop the reaction; or add the iodine solution at once without this preliminary step, and read the results. Add a few drops of iodine solution to each tube.

Complete starch digestion is indicated by a clear solution with only the brown iodine color showing. The tubes containing undigested starch give a dark blue color.

The end point is seen by noting that the intermediate tubes have a deep red color, indicating that hydrolysis has progressed only to the stage of dextrin. The last tube having a predominantly red color is accepted as showing diastase activity, and is chosen as the end point.

If digestion is complete in all tubes, dilute the urine further: 1 c.c. of the 1:30 dilution to 9 c.c. of water, making a dilution of 1:300, and proceed with the test.

The diastase number (an arbitrary unit of measure) is calculated as follows:

$$\frac{\text{Dilution of urine} \times \text{c.c. of starch solution} \times \text{number of drops per c.c. delivered by the pipette}}{\text{Number of drops of urine in the final tube}}$$

Example:

Digestion is complete in the tube with 1 drop of urine; pipette delivers 20 drops per c.c. Dilution of urine, 30.

$$\frac{30 \times 2 \times 20}{1} = 1200 \text{ units.}$$

A diastase number of 300 or less is within **normal** limits when this test is used.

Interpretation.—

The values in patients with proved acute pancreatitis may vary from 600 to 20,000 units. The interpretation is the same as that of serum amylase except that elevated values may be detected over a longer period in the urine than in the serum. Some investigators believe that examination of urine is preferable to that of the blood.

Preformed Creatinine (Folin)**Reagents.—**

These are the same as for the creatinine in blood, page 271.

Principle.—

Creatinine reacts with picrates in alkaline solution, to give, at room temperature and within a few minutes, an intense red color that is stable and can be read in a colorimeter.

Equipment.—

- 1 100 c.c. graduated cylinder.
- 1 100 c.c. volumetric flask.
- 2 1 c.c. pipettes graduated in 0.1 c.c.
- 1 20 c.c. volumetric pipette.

Technic.—

Make an alkaline picrate solution by adding
10 c.c. of 10% sodium hydroxide solution
to 50 c.c. of saturated solution of picric acid.

Into a 100 c.c. volumetric flask marked "U" for unknown place:
1 or 2 c.c. of urine.

Into a 100 c.c. volumetric flask marked "S" for standard place 1 c.c. of stock solution of creatinine, Folin (1 c.c. contains 1 mg. creatinine).

To both standard and unknown add 2 c.c. alkaline picrate solution.

Let stand ten minutes.

Dilute both to 100 c.c. with distilled water.

Mix, and read in the colorimeter.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 1 \times \frac{\text{Volume in 24 hours}}{\text{c.c. urine used}} = \text{mg. creatinine in 24 hours.}$$

Example.—

Reading of standard is 15; reading of unknown is 10: 1 is the strength of the standard solution; 1500 c.c. urine in the 24-hour specimen; 2 c.c. urine used.

$$\frac{15}{10} \times 1 \times \frac{1500}{2} = 1125 \text{ mg. preformed creatinine in 24 hours, or 1.125 gm.}$$

Normal is about 1 to 1.5 gm. in 24 hours.

Urea Nitrogen, Folin

Reagents.—

These are the same as for urea nitrogen in blood, page 247.

Equipment.—

- 1 small test tube ($\frac{5}{8} \times 5$ inches).
- 4 1 c.c. pipettes graduated in 0.1 c.c.
- 1 medium-sized test tube.
- 2 100 c.c. lipless cylinders.
- 1 5 c.c. volumetric pipette.
- 1 20 c.c. volumetric pipette.
- 2 2 c.c. volumetric pipettes.
- 1 3 c.c. volumetric pipette.
- 1 10 c.c. volumetric pipette.

Technic.—

Place 9 c.c. distilled water in a test tube.

Add 1 c.c. urine, and mix. This gives a 1:10 dilution of the urine.

Place 1 c.c. of the diluted urine in a test tube that will readily slip into a 100 c.c. graduated lipless cylinder.

Add 0.5 to 1 c.c. urease solution. (Add 1 to 2 drops of pyrophosphate solution if urease from any firm other than Squibb, Arlington, or Hynson, Westcott, and Dunning is used.)

Place in a water bath at 50 to 55° C. for five minutes or at room temperature for fifteen minutes to convert the urea to ammonium carbonate.

Add 1 c.c. antifoaming mixture, or Mallinckrodt's fusel oil.

Add 5 c.c. saturated solution of sodium carbonate under the solution.

Place in cylinder No. 2 for aeration (see page 248), and adjust stopper so that the end of the delivery tube reaches the bottom of the test tube. Close tightly.

In a graduated lipless cylinder, marked "No. 1," place 15 to 20 c.c. distilled water.

Add 2 c.c. N/10 hydrochloric acid, and close, making sure that the end of the delivery tube reaches the bottom of the cylinder.

Attach to the suction pump, placing the acid wash bottle at the end. (See page 249.)

Start the suction and continue aeration for thirty to forty-five minutes.

The contents of cylinder No. 1 are used for the final determination.

In a 100 c.c. volumetric flask, marked "S" for standard, place 3 c.c. standard ammonium sulphate solution, Folin (1 c.c. contains 0.1 mg. nitrogen).

Add 70 c.c. distilled water.

To cylinder No. 1 (unknown) add distilled water to the 22.5 c.c. mark.

Add 2.5 c.c. Nessler's reagent (diluted Nessler's solution, Folin).

To the standard in the volumetric flask, add 10 c.c. Nessler's reagent.

Dilute the standard to 100 c.c. with distilled water and mix.

Dilute the unknown to 25, 50, 75, or 100 c.c. with distilled water, depending on the depth of color. The color must be lighter than the standard.

Mix, and read immediately in the colorimeter.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{\text{Dilution of Unknown}}{\text{Dilution of Standard}} \times 0.3 \times \frac{\text{Vol. of 24-hour specimen}}{\text{c.c. urine used (0.1)}} = \text{the urea nitrogen mg. in 24 hours. To convert to urea, multiply this figure by 2.14.}$$

Example.—

Reading of standard is 15; reading of unknown is 20; dilution of unknown is 100; dilution of standard is 100; 0.3 is strength of standard in mg.; 1500 c.c. urine in the 24-hour specimen; 0.1 c.c. urine used.

$$\frac{15}{20} \times \frac{100}{100} \times 0.3 \times \frac{1500}{0.1} = 3,375 \text{ mg. or } 3.375 \text{ grams in 24 hours.}$$

To convert to urea, multiply 3.375 grams urea nitrogen by 2.14 equals 7.2225.

Normal urea is about 10 to 40 gm. in 24 hours.

Ammonia Nitrogen, Folin

Reagents.—

These are the same as for urea nitrogen in blood, page 247.

Equipment.—

- 2 100 c.c. lipless cylinders.
- 1 medium-sized test tube.
- 3 2 c.c. volumetric pipettes.
- 3 1 c.c. pipettes graduated in 0.1 c.c.
- 1 set aeration equipment described on page 248.
- 1 20 c.c. volumetric pipette.
- 1 3 c.c. volumetric pipette.
- 1 10 c.c. volumetric pipette.

Technic.—

For this test, if the urine is very dilute, use 5 c.c. urine instead of 2 c.c.

Place 2 c.c. urine in a test tube that will slip readily into a 100 c.c. lipless cylinder.

Add 1 c.c. antifoaming mixture, or Mallinckrodt's fusel oil.

Allow 4 c.c. saturated solution of sodium carbonate to run under the solution. (If 5 c.c. urine are used, use 7 c.c. sodium carbonate.)

Carry out the aeration and development of color as described on page 142, urea nitrogen in urine, method of Folin.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{\text{Dilution of Unknown}}{\text{Dilution of Standard}} \times 0.3 \times \frac{\text{Vol. of 24-hour specimen}}{\text{c.c. urine used}} = \text{the mg. ammonia nitrogen in 24 hours.}$$

Example.—

Reading of standard is 15; reading of unknown is 20; dilution of unknown is 100; dilution of standard is 100; 0.3 is mg. nitrogen in the standard; 1500 c.c. urine in 24 hours; 2 c.c. urine used in the test.

$$\frac{15}{20} \times \frac{100}{100} \times 0.3 \times \frac{1500}{2} = 168.75 \text{ mg. ammonia nitrogen in the 24-hour specimen.}$$

To convert to ammonia, multiply by 1.214.

Normal.—About 600 mg. of ammonia N are excreted in 24 hours. This quantity is increased in acidosis or by eating acid-forming foods. It is decreased by ingestion of alkali.

Uric Acid in Urine, Benedict and Franke

Reagents.—

The arsenophosphotungstic acid, sodium cyanide solution, and stock solution of uric acid are the same as are used for the estimation of uric acid in blood employing the method of Benedict with Folin filtrate.

Standard Solution of Uric Acid for Use (1 c.c. = 0.02 mg.)

Dilute 25 c.c. of the stock solution (page 266) to about 200 c.c. with distilled water in a 250 c.c. volumetric flask.

Add 12.5 c.c. of 10% hydrochloric acid.

Mix and dilute to volume with distilled water.

Equipment.—

- 1 100 c.c. graduated cylinder.
- 2 50 c.c. volumetric flasks.
- 2 10 c.c. volumetric pipettes.
- 2 burets.

Technic.—

Dilute 1 c.c. urine in a cylinder to 15, 20, 25 c.c. and record the dilution.

Pipette 10 c.c. of the diluted urine into a 50 c.c. volumetric flask labeled "U" for unknown.

Pipette to a similar flask labeled "S" for standard 10 c.c. of the standard solution (contains 0.2 mg. uric acid).

Add from the buret 5 c.c. of 5% sodium cyanide solution to each. DO NOT PIPETTE!

Add from a buret 1 c.c. of arsenophosphotungstic acid. POISON!

Shake gently to mix and allow to stand 5 minutes.

Dilute each to 50 c.c. with distilled water.

Compare in a colorimeter.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.2 \times 10 \times \text{dilution} = \text{mg. uric acid in 100 c.c. urine.}$$

To determine the amount in 1500 c.c. multiply by 15.

Example.—

Reading of standard is 20; reading of unknown is 35; dilution was to 20.

$$\frac{20}{35} \times 0.2 \times 10 \times 20 = 22.8 \text{ mg. uric acid in 100 c.c. urine.}$$

$22.8 \times 15 = 342 \text{ mg. or } 0.342 \text{ gm. in 1500 c.c.}$

Normal—0.2 to 2 gm. in 24-hour specimen of 1500 c.c.

Total Nitrogen, Folin

Reagents.—

These are the same as for total nonprotein nitrogen in blood, page 244.

Equipment.—

- 1 100 c.c. graduated cylinder.
- 1 5 c.c. volumetric pipette.
- 2 1 c.c. pipettes graduated in 0.1 c.c.
- 1 Pyrex test tube (1 × 8 inches).
- 1 microburner, ringstand, and test tube clamp.
- 2 200 c.c. volumetric flasks.
- 1 10 c.c. volumetric pipette.
- 1 2 c.c. volumetric pipette.
- 1 15 c.c. volumetric pipette.

Technic.—

Dilute 5, 10, or 20 c.c. urine to 100 c.c. with distilled water and mix.

Place 1 c.c. diluted urine in a Pyrex tube.

Add 1 c.c. digestion mixture.

Heat over a microburner until the solution turns black, then clear again. (See page 245 for description.)

Cool less than two minutes.

Rinse the digested mixture into a 200 c.c. volumetric flask with 125 c.c. distilled water.

Into a 200 c.c. volumetric flask marked "S" for standard,

Place 10 c.c. standard ammonium sulphate solution, Folin (containing 1 mg. nitrogen).

Add 2 c.c. digestion mixture.

Add 125 c.c. distilled water.

Give both flasks a whirl, and add

30 c.c. Nessler's reagent (diluted Nessler's solution, Folin) to each.

Shake both flasks.

Dilute both to 200 c.c. with distilled water.

Mix and read in the colorimeter.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{\text{Volume of 24-hour specimen}}{\text{c.c. urine used}} = \text{mg. total nitrogen in 24 hours.}$$

Example.—

Reading of standard is 15; reading of unknown is 20; volume in 24 hours is 1500 c.c.; 0.1 c.c. urine used (10 c.c. urine, diluted to 100 c.c., and 1 c.c. of this used in the test).

$$\frac{15}{20} \times \frac{1500}{0.1} = 11250 \text{ mg., or 11.25 grams total nitrogen in 24 hours.}$$

Normal for adults is 12 to 18 gm. in 24 hours.

Chlorides, Volhard-Arnold*

Reagents.—

Standard Silver Nitrate Solution for Urine Chlorides (1 c.c. reacts with 0.01 gram sodium chloride, or 1 c.c. standard ammonium thiocyanate solution, for urine chlorides).

Dissolve 29.06 grams silver nitrate crystals in a small amount of distilled water in a 1000 c.c. volumetric flask.

Dilute to 1000 c.c. with distilled water and mix. Store in a brown bottle.

Standard Ammonium Thiocyanate Solution for Urine Chlorides (1 c.c. reacts with 1 c.c. standard silver nitrate solution for urine chlorides, or 0.01 gram sodium chloride).

Dissolve 13 grams ammonium thiocyanate in 800 c.c. distilled water.

Titrate against the silver nitrate solution using 1 c.c. ferric alum indicator, until each c.c. of this solution equals each c.c. of the silver nitrate solution.

See page 433 for a full description.

Ferric Alum Indicator.

Dissolve 100 grams crystalline ferric ammonium sulphate in 100 c.c. of 25% nitric acid.

Equipment.—

- 1 small casserole or evaporating dish.
- 1 5 c.c. volumetric pipette.
- 1 20 c.c. volumetric pipette.
- 1 10 c.c. volumetric pipette.
- 1 2 c.c. volumetric pipette.
- 1 buret.

Technic.—

Place 5 c.c. urine into a small casserole or evaporating dish.

Add 20 c.c. distilled water.

Add 10 c.c. standard silver nitrate for urine chlorides (1 c.c. reacts with 0.01 gram sodium chloride).

Add 2 c.c. ferric alum indicator.

*Refer to Blood Chemistry chapter for other methods.

Mix, and titrate by running in standard ammonium thiocyanate for urine chlorides (1 c.c. is equivalent to 0.01 gram sodium chlorides, or 1 c.c. of the standard silver nitrate solution used), from a buret until the first trace of yellow shows throughout the mixture on stirring.

This is the end point. Read from the buret the number of c.c. of standard ammonium thiocyanate used.

Calculation.—

Subtract the number of c.c. of ammonium thiocyanate used from 10.

Multiply this difference by 0.01 to obtain the grams of sodium chloride in 5 c.c. urine. Multiply this figure by the factor for the 24-hour volume.

Example.—

1500 c.c. urine in the 24-hour specimen; 5 c.c. urine used in the test; 6.2 c.c. standard ammonium thiocyanate used.

$10 - 6.2 = 3.8 \times 0.01 = 0.038$ gram sodium chloride in 5 c.c. urine. In 1500 c.c. there will be 300 times 0.038 gram, or 11.4 grams sodium chloride.

Normal number of grams of chlorides, as sodium chloride, is 10 to 15 in 24 hours.

To convert to meq./L., use the formula on page 415.

Phosphates in Urine

Reagents.—

Special Sodium Acetate Solution.

Dissolve	100 grams sodium acetate
in	800 c.c. distilled water in a liter volumetric flask
Add	100 c.c. of 30% acetic acid
Dilute to	1000 c.c. with distilled water.

Uranium Nitrate Solution.

Dissolve	44.8 grams uranium nitrate
in	900.0 c.c. distilled water, then titrate with a standard phosphate solution containing 0.005 grams of P_2O_5 per c.c., and make the necessary adjustment.

Standard Phosphate Solution.

Dissolve	14.721 grams of pure air-dry sodium ammonium phosphate ($NaNH_4HPO_4$ plus $4H_2O$)
in about	800.0 c.c. distilled water and
dilute to	1000.0 c.c. with distilled water.

10% Potassium Ferrocyanide Solution.

Dissolve 10 grams of potassium ferrocyanide in a small amount of distilled water, and dilute to 100 c.c. with distilled water.

Equipment.—

- 1 liter beaker or Erlenmeyer flask.
- 1 5 c.c. volumetric pipette.
- 1 buret.
- 1 porcelain tablet.

Technic.—

Place 500 c.c. urine in a beaker or Erlenmeyer flask.

Add 5 c.c. special sodium acetate solution.

Heat to the boiling point.

Add standard uranium nitrate solution drop by drop from a buret until no further precipitate forms.

Place one drop of the mixture on a porcelain tablet.

Add 1 drop of a 10% solution of potassium ferrocyanide.

A brownish-red color should form. If this color does not appear, add more uranium nitrate standard solution until the mixture is brownish-red.

Read from the buret the number c.c. uranium nitrate used.

Calculation.—

No c.c. uranium nitrate solution used $\times 0.005 \times \frac{\text{volume in 24 hours}}{50} = \text{grams of } P_2O_5$
in 24 hours.

Example.—

1500 c.c. urine in the 24-hour specimen; 50 c.c. urine used;

10.2 c.c. uranium nitrate solution used in the titration.

$$10.2 \times 0.005 \times \frac{1500}{50} = 1.53 \text{ grams } P_2O_5 \text{ in 24 hours.}$$

Normal P_2O_5 is 1 to 2 grams in 24 hours.

Precipitation of Phosphates by Ferric Chloride

Add 10 drops of 10 per cent ferric chloride to 10 c.c. of urine.

A white precipitate occurs. This precipitate may be dull gray, red, dark to ink black.

“This variation of color which ferric chloride gives to urine depends upon quantitative and qualitative variations in the composition and hydrogen ion concentration of the specimen. Thus, for example, at normal pH, phosphates will give light colored precipitates, whereas in urine with neutral or slightly alkaline reactions, especially when the phosphate concentration is low, darker colored precipitates will be formed. Black precipitates may result from the presence of small amounts of tannin with which the urine may be contaminated from extraneous sources. Unusually large quantities of phenolic compounds tend to impart dark coloration to the precipitate.”*

Estimation of Bismuth in Urine

Other tests for bismuth in urine are given in Chapter XV.

A rapid clinical method for estimation of bismuth in urine has been worked out by Hanzlik, Lehman, Richardson and Van Winkle.¹

This method utilizes the principles of direct oxidation by potassium permanganate, sulphuric acid and heat, decolorization with oxalic acid to the clearness of water, and of a reducing agent to prevent oxidation of sodium iodide for reaction with bismuth in a strongly acid solution. The iodide reacts with bismuth to form sodium iodobismuthite, a colored ion which permits direct colorimetric estimation by matching with permanent color standards on a paper scale.

The equipment consists of a small iron support, a clamp, a Pyrex test tube, 20 \times 3 cm., and a microburner. It is necessary to guard against foaming in the large test tube by playing the flame of the microburner up and down the sides of the test tube. The paper scale was prepared by Mr. George H. Needham, of San Francisco, and is obtainable from the James H. Barry Co., 170 Van Ness Avenue, South, San Francisco. Six color standards, indicating quantities of bismuth ranging from 0.25 to 1.2 mg. per hundred cubic centimeters, are represented on the paper scale. Intermediate quantities of bismuth may be estimated by interpolation. A legend below the color standards gives the following information: milligrams of bismuth per hundred cubic centimeters, daily output of bismuth in 1,200 c.c. of urine (when the daily volume is known an estimation can be made accordingly), and the details of the procedure for testing urine.

The bismuth is estimated in six steps as follows:

1. Place 10 c.c. of urine in a long test tube, 3 \times 20 cm.

2. Add 0.4 gm. of potassium permanganate and 2 c.c. concentrated sulphuric acid.

Heating will produce foaming.

3. Boil gently over a microburner for two minutes.

4. Add 0.4 gm. of oxalic acid. Decolorization will take place, and the solution should be allowed to cool.

*Personal communication by Dr. Michael Somogyi, St. Louis, Mo.

¹Hanzlik, P. J., Lehman, A. J., Richardson, A. P., and Van Winkle, W., Jr.: Arch. Dermat. & Syph. 36: 725, 1937.

5. Add from 0.01 to 0.04 gm. of sodium sulphite and sodium sulphate and 0.05 gm. of sodium iodide. The fluid will become yellowish green if bismuth is present.

6. Match with the color scale, holding the test tube against the white margin above the standards.

If for an occasional specimen complete oxidation does not take place, indicated by the presence of some tint, the procedure from the second step should be repeated.

The final oxidized solution must be clear as water for the proper matching of colors.

The reagents mentioned above, with the exception of the concentrated sulphuric acid, are obtainable in tablet form. Instead of the quantities mentioned in steps 2, 4, and 5, one tablet may be used containing the quantity needed in the steps.

CYSTOSCOPIC URINALYSIS

Special consideration must be given to analyses of urine in connection with cystoscopic examinations. The method here recommended will obviate much of the irritation on the part of urologists who send cystoscopic urine specimens to laboratories and fail to receive the information which is very important in connection with their urologic examinations. These points have been so very well stressed by E. A. Fennel,¹ of Honolulu, T. H., that the following résumé of his article on cystoscopic urines is reproduced.

There must be some cooperation between the urologist and the clinical pathologist before the examination, so that the maximum amount of information may be obtained from the urinalysis. Preliminary information should be given to the laboratory as to whether the preliminary tentative diagnosis is bladder neoplasm, unilateral or bilateral tuberculous nephritis, nonspecific bacterial pyelonephritis, simple prostatic hypertrophy, or nephrolithiasis. With that information, the pathologist may proceed to an examination which will afford the urologist the maximum of information and satisfaction. Fennel's technic is as follows:

Urine from right ureter, left ureter, and bladder is usually similarly analyzed. *Color* of a single specimen may be unimportant, but relative color of the three specimens may be significant; for example, if the bladder urine is clear amber, and the ureteral urine is red and turbid, suspect trauma to ureters. Erythrocytes in the latter may have little or no additional significance.

Appearance, usually unimportant, should be noted. Occasionally diagnosis of unilateral lymphatic obstruction may result from observation of a unilateral opalescence, although this may be so slight that the proof of chyluria is achieved only with great difficulty. Dr. Fennel stated that in nearly 20 years of practice in Hawaii he had not seen a single native authenticated case of filariasis, the chyluria being usually idiopathic or traceable to a renal or perirenal obstruction.

Presence and amount of **albumin** may be significant if blood extravasation due to trauma may be ruled out. Robert's reagent is used, quantitative estimations being made upon the request of the urologist. The quantitative method is a modification of the Kingsbury, Clark, Williams, and Post² technic. The standards signify varying quantities of serum albumin; they are permanent for many months.

Technic.—

Standards.—Make stock suspension by adding 0.5 c.c. saturated alcoholic solution of gum mastic to 4.5 c.c. of 95% alcohol. Add 10 c.c. distilled water, drop by drop, shaking constantly.

¹Fennel, E. A.: Proceedings of the Staff Meeting of the Clinic, Honolulu, T. H. 4: May, 1938.

²Kingsbury, F. B., Clark, C. C., Williams, G., and Post, A. L.: Rapid Determination of Albumin in Urine, J. Lab. & Clin. Med. 11: 981, 1926. (See p. 62.)

Rack up ten tubes. Add the milky stock suspension in the amounts of 1.0, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, and 0.025 c.c., respectively. Add enough distilled water to each tube to bring the total in each to 1.0 c.c. Add to each tube 10 c.c. of 2 per cent agar (Difco), and 0.3 c.c. of 1:10 formalin. Mix contents of each tube thoroughly and allow to harden in ice water. These tubes represent mg. of albumin per 100 c.c. as follows: 125, 100, 75, 62.5, 50, 37.5, 25, 12.5, 6.25, and 3.12. One c.c. of blood serum in 100 c.c. water is directly comparable with the 50 mg. standard tube.

Method.—Mix 1.25 c.c. fresh urine with 3.75 c.c. of 3% sulphosalicylic acid and compare in a slanting light with the standards for equal turbidity.

Sugar, although listed on cysto-urine reports, is rarely done. If the bladder urine is negative, it is unnecessary to test the ureteral urine for sugar.

Reaction of urines, in terms of pH, is very important with respect to investigations concerning precipitation of stones and effectiveness of certain urinary antiseptics. Chemical cleanliness of the cystoscope and ureteral catheters is very important in this estimation, as is the method of sterilization. The free alkali of the glass of urine containers must be considered. If possible, determine the pH electrometrically, particularly if the specimens are heavily buffered by albumins. Colorimetric methods are satisfactory from the clinical point of view. Use phenol red, bromthymol blue, and methyl red for a range from 8.4 to 4.4. It is extremely important to compare the pH of the three different specimens of urine if interfering factors which influence the pH are to be ruled out. An inflammatory process in the kidney usually makes for a more acid urine; bacterial growth in that urine may, however, alter the picture.

Quantity may be significant if collection time has been bilaterally equal, in which case it is really a function test. Otherwise it is estimated simply for the record.

Other chemicals are seldom needed.

Microscopic Examination.—

The laboratory and urologist must have complete understanding if the microscopic findings are to be properly estimated and interpreted. Report the actual number of formed elements per low- or high-power field on a ++++ basis; be sure the urology department understands just what is meant by a +, ++, +++, and ++++.

Epithelia may or may not be important. Differentiate between the large squamous type, coming from the bladder, and the smaller, often pear-shaped cell, coming from the ureter. Presence of many of the latter type in the bladder before catheterization may indicate a moderately mobile ureteral stone (even without blood), or a very mild pyelitis. Differentiation between ureteral epithelia and those from the pelvis of the kidney is extremely difficult, and unnecessary; call these two "renal" in contradistinction to "bladder" epithelia.

The identification of isolated neoplastic epithelial cells is often not possible. When small fragments of adherent cells are obtained, in which some degree of architecture remains, an opinion may be advanced. When the fragments are tiny, crush them between two slides in polychrome methylene blue (like trichina in muscles) and examine. If large enough, prepare a section in the usual manner. (The urologist must not expect too much. The tip of a papilloma may permit of identification as a papilloma, but permits of no expression on its malignancy—only the pedicle and attachment make that possible. The urologist draws his own conclusions as to a possibility of a bladder papilloma.)

Leukocytes are examined in the wet specimen, and later stained. Report the number in a centrifuged specimen on a ++++ basis. Clumping is as important as actual numbers. Large aggregations may mean active productive inflammation; smaller groups, chronicity or mildness; small numbers diffused throughout the specimen may be without significance. In acute conditions cells are small, compact, opaque; in subacute conditions, they are larger, swollen, and have a glassy refractility, with a reduction in numbers; in healing and reparatory stages, they become very hydropic, large and clear, with many very large granular macrophages which act as scavengers of particulate matter and bespeak a good prognosis.

Erythrocytes are very significant if the cystoscopist can vouch for the gentleness of his manipulations. The actual number of these cells in a bloody urine is very important.

Total absence of red blood cells indicates low hydrogen ion concentration and careful technic, or a careless technician; most urines from normal persons will show a few erythrocytes if carefully examined. With a pH of 4.4 or lower in a healthy person, 2, 3, or 4 red blood cells per low-power field in a centrifuged specimen is usually found; they have been extruded from an intact capillary. Occult blood reactions are not so sensitive as the microscope. If the 4 or 5 cells in occasional fields are seen in a sort of rouleaux formation, it may indicate a break in continuity of the epithelium, such as may be caused by a very slightly mobile stone.

Amorphous phosphates and urates and crystals are noted as a possible aid in diagnosing the type of lithiasis, although the value of such aid is dubious. Even sectioning and chemical analysis of extirpated stones has been of little value, since the majority of these stones are laminated and composed of a variety of different substances. Only occasionally is a stone of relative purity found, such as phosphate or uric acid. See page 117 for optical analysis of urinary calculi.

Casts are studied; the type of cast is more important than the number. A cellular cast may be produced in a very short time, while a hyaline cast has probably lain in the urinary tubule a very long time. A kidney containing numberless casts, but secreting an acid urine of pH 4.4, or lower, may release very few into the urine. Such a kidney is a hydrophilic colloid in a rigid capsule; in the presence of much acid it swells with the absorption of water within the cells, and so compresses its own tubules. Really massive doses of alkali, sufficient to make the urine neutral, frees the water, decompresses the kidney, and so releases showers of mobilized casts, the first to appear being hyaline. Cellular or blood casts speak for the acuteness of the nephritis.

Examine unstained wet preparations for presence or absence of **bacteria**, their quantity, motility, morphology. Make smear preparations of large drops of urine from the left ureter, right ureter, and bladder on each of two slides. Use one for the wet preparation; stain the other. Use a small drop of Mayer's albumin fixative on the slide if there is no albumin in the urine. Air dry rapidly and leave in the paraffin oven at 56° C. overnight. Stain without further fixation. If the urologist requests an examination for tuberculosis, use the Ziehl-Neelsen stain. All acid-fast stains are made on new slides. Since these are catheterized specimens, the smegma bacillus and other acid-fast saprophytes are of no practical significance. Search intensely with a mechanical stage; if the examination is carried out properly, it may yield as much information as culture or guinea pig inoculation.

Urines of pH 4.4 or lower, with a fair trace of albumin, moderate number of leukocytes, easily found erythrocytes, few or no contaminating organisms, are more apt to show tubercle bacilli than those which are alkaline or neutral, with many leukocytes, few erythrocytes, and heavy bacterial flora. Fennel found that the positives by culture or guinea pig were so few over and above those found by direct bacterioscopic examination, that he considers the culture and guinea pig useless for practical purposes. (Even with sputums, he has found that with ten consecutive negative sputum examinations, the culture or guinea pig inoculations yield very little if any additional information.) Only 5 to 7 per cent of negative bacterioscopic urines were positive by culture, while an additional 3 per cent were positive by guinea pig inoculation. The culture gives better results than the guinea pig inoculations.

Cultures.—Centrifuge separately the three specimens of urine (left ureter, right ureter, bladder) in sterile corked tubes. Use the supernatant fluid for chemistry. Use the sediment for smears and cultures. Refer to the chapter on Bacteriology.

Bacteriologic examinations are equally applicable to voided bladder urines. In the males, carefully cleanse the meatus, collect the voided urine in two containers, discarding the first specimen. In females, the meatus is cleansed and a tube is used. This tube is a test tube with lip, with the bottom cut off, the tube being bent at a slight angle at the middle. The nurse holds this over the meatus and collects the urine in two containers. This tube method is also useful in collecting urine during menstrual periods.

Functional Tests.—Formerly too much stress was laid on functional tests, particularly the phenolsulphonephthalein test. Only if this dye is given in accurate dosage, intravenously, is the test of value. Subcutaneous or intramuscular injections introduce the potent factor of absorption into the calculations. A subcutaneous edema may retard absorption, giving un-

reliably low results. The best index to the combined function of both kidneys is blood chemistry and the retention of nitrogenous products. When a partition of the estimated renal function of both sides is desired, phenol red and indigo carmine are useful tests. By the injection of material for pyelography and interval plates, not only is a picture of the renal pelvis obtained, but also an estimate of the time and quantity of excretion of each kidney. Fennel uses Diodrast for pyelography.

Since sulfanilamide is almost wholly excreted by the kidneys, it may become a good means of testing renal function, after the normal is established. Normally all sulfanilamide is removed from the blood within 48 hours. Fennel observed a case of congenital atresia of the kidneys in a girl aged 18 years, in whom a pyelonephritis led to imbalance and nitrogen retention. Eight days after a small dose of sulfanilamide had been given therapeutically, fair traces of the drug could still be found in her blood. She died soon afterward with symptoms of uremia.

ESTIMATION OF KIDNEY FUNCTION

There are a number of tests for the estimation of kidney function. Before proceeding to outline the manner of performance of these tests, some fundamental facts regarding the physiology of the kidney must be reviewed. For those who desire an exhaustive and timely knowledge of the true physiology of the kidney, it is suggested that the textbook of Homer W. Smith, *The Physiology of the Kidney*, Oxford University Press, be carefully read. This work gives a complete outline of the physiology as it is construed by modern writers. Attention is to be called to the diagram from Smith's book (Fig. 72) showing the essential features of the typical nephron in the human kidney. This anatomical study of the nephron or unit of kidney structure shows the glomerulus in which the formation of urine begins. The glomerulus is a tuft of capillaries supplied with blood through an afferent arteriole. The capillary tuft is thrust into the expanded but closed end of the tubule so far that the tuft has come to be enveloped by a double layer of tubular epithelium; the inner or visceral layer being closely applied to the capillaries, extending in between all the loops and surrounding each loop almost completely; the outer or parietal layer forming a smooth spherical capsule, Bowman's capsule, about the tuft of capillaries. Any fluid passing through the capillaries enters the capsular space around the tubule. This is lined with a thin glomerular basement membrane, anatomically continuous with the basement membrane bounding the outer surface of the tubule cells. The tubule shows a convolution near the glomerulus, known as the proximal convoluted tubule, passes by a more or less straight course towards the pelvis of the kidney, and abruptly reverses its direction, forming what is commonly called Henle's loop. It then returns to the region of its own glomerulus where it undergoes the second series of convolutions, the distal convoluted tubule, and later joins a collecting duct. The proximal and distal convoluted segments of one tubule are closely intertwined in one locality.

It is generally believed at this time that the process which takes place in the glomerulus is pure filtration. The capsular fluid contains all the filtrable constituents of blood plasma in the same concentration per unit volume of water as these are present in the plasma, according to Smith, "except for such inequalities in the distribution of ions as might arise from the presence of non-filtrable proteins."

We know that the substances which are present in the plasma but absent from the urine must be reabsorbed by the tubules as the glomerular filtrate passes along them on its way to the bladder. A large portion of the water present in the filtrate is also reabsorbed by the tubules. This is evidenced by the fact that products such as creatinine and urea may be present in the urine in many times the concentration that they are present in the plasma. According

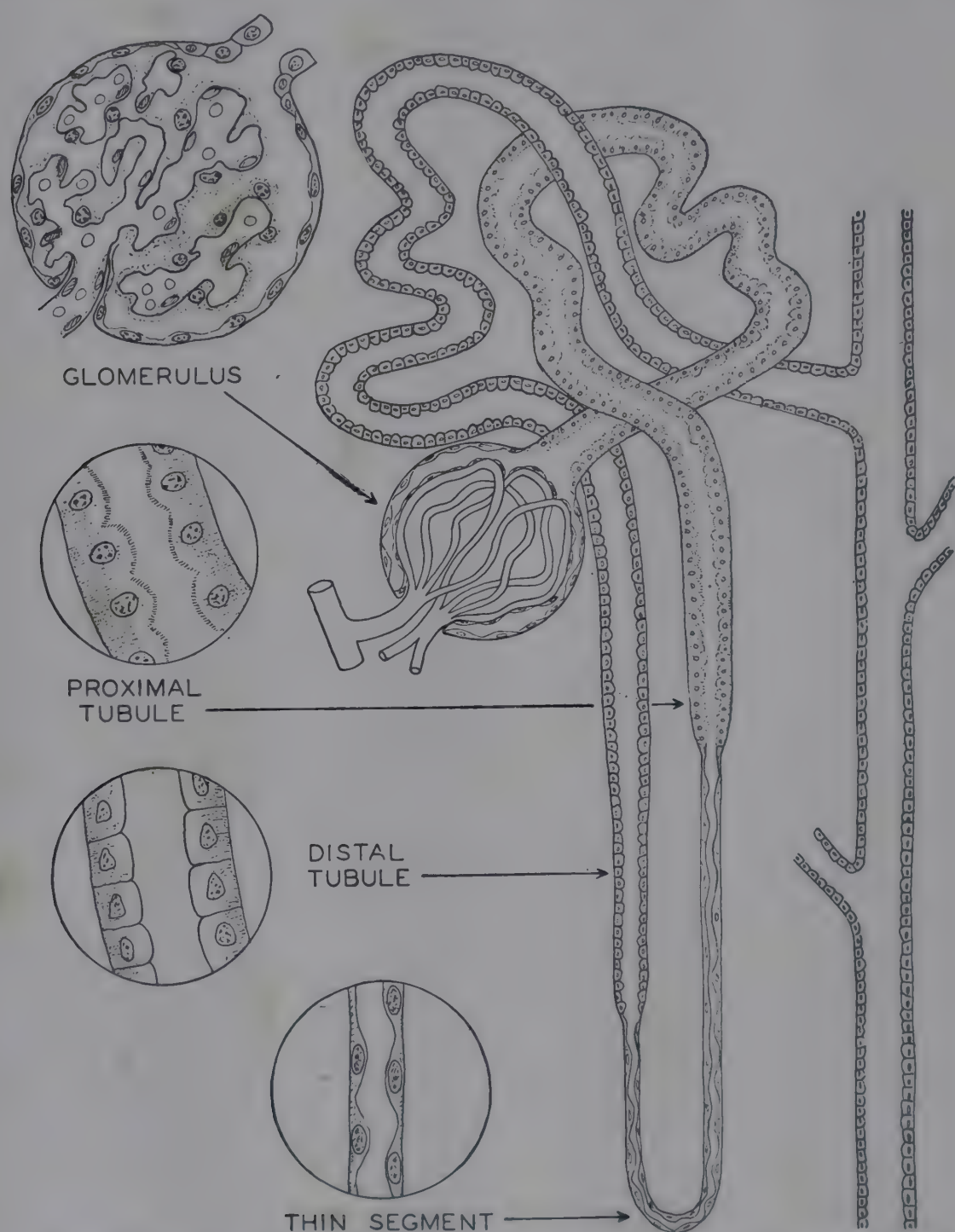


Fig. 72.—Diagram showing the essential features of a typical nephron in the human kidney. (From Smith, H. W.: *The Physiology of the Kidney*, Oxford University Press.)

to Smith, in every man about 90 c.c. of filtrate are formed in the kidneys every minute, of which 89 c.c. of water are reabsorbed by the tubules, leaving 1 c.c. of urine to be excreted, even under conditions of relatively high urine flow. Variations in urine flow are referable primarily to variations in the amount of water reabsorbed rather than to the variations in the amount of filtrate formed.

Some consider that the glomerulus is a filter for urea nitrogen and therefore tests for identifying urea nitrogen in blood and urine are tests of glomeru-

lar function. The proximal convoluted tubule must be considered as that part of the kidney structure which is concerned with the reabsorption and elimination of other components of urine.

Tests for concentration of urine are therefore used to demonstrate the function of the proximal convoluted tubules of the kidney. There are a number of physiologic factors in connection with these two elementary principles. First, the glomerular blood pressure is of importance in relation to effective filtrate pressure. From a number of experimental observations, it has been shown that increase of pressure in the renal circulation causes corresponding increase in urine formation. This supports the evidence derived from anatomy that the glomerulus is the structure in which changes in pressure operate to cause changes in urine volume. Furthermore, the contractility of the efferent vessel is of importance in regard to the regulation of glomerular filtration. Another factor involved in the maintenance and adjustment of glomerular capillary pressure and associated with the filtration of cell-free, protein-free fluid from the blood as it flows through the glomerular capillaries is viscosity. The number of cells per unit volume in the blood in the efferent arteriole is higher than that in the afferent. This is a factor which must vary with the rate of blood flow, volume of glomerular filtrate, and caliber of vessels.

The function of the proximal tubule is normally bound up with the process of reabsorption. Wearn and Richards¹ first showed by puncture of the capsule of Bowman that the urine from that portion contained sugar and chlorine, while urine issuing from the ureter contained none: hence the principle of reabsorption was established. At the same time, Bieter and Hirschfelder² showed that when they injected phenol red into frogs the contents of the tubules were more intensively stained than were the contents of the capsule of Bowman. This observation was presented by them as reabsorption of water from the tubules. From experiments with phenol red, it was shown that it is a substance the excretion of which cannot be accounted for by glomerular filtration. In other words, this substance which cannot be diffused through the walls of the tubule must be secreted in the proximal portion.

Expressed in other terms, the glomerular portion of the kidney is able to eliminate practically all soluble and diffusible substances which may enter the blood stream. This process is in line with the filtration theory of glomerular function. The evidence is definite that reabsorption takes place in the proximal portion of the tubule. Urinary secretion consists of glomerular filtration, tubular reabsorption, and tubular secretion, with possible evidence of some amount of glomerular secretion. According to Berglund and others,³ the formation of the urine is effected through the following processes:

“1. Ultrafiltration of large amounts (for the human kidney, about 150 c.c. per minute) of protein-free fluid in the glomeruli.

“2. Active reabsorption of water.

“3. Active reabsorption of threshold bodies, like sugar and chlorides.

¹Wearn, J. T., and Richards, A. N.: *Am. J. Physiol.* 71: 209, 1924.

²Bieter, R. N., and Hirschfelder, A. D.: *Am. J. Physiol.* 68: 326, 1924.

³Berglund, Medes, Huber, Longcope, and Richards: *The Kidney in Health and Disease*, p. 78, Philadelphia, 1935, Lea & Febiger.

“4. Passive back diffusion of all other substances in degrees varying according to the permeabilities of the tubule walls for each—with creatinine at one end as the most highly concentrated and alcohol at the other end as a substance not concentrated at all.

“5. Secretion of substances not preformed in the blood.”

Regarding the excretion of sugar in the urine, it has been known for a long time that whenever the quantity of glucose in the blood increases, sugar is likely to be found in the urine. This occurs when there is failure on the part of the blood to metabolize carbohydrates. Sugar filters out through the glomerulus, and its presence in the urine must be due to the fact that the tubules fail to reabsorb it rather than to any impairment of renal function. The threshold point for sugar is generally supposed to be around 170 mg. per cent, although the author has seen it as high as 210 mg. per cent. In other words, the threshold point in man is variable. In renal diabetes the threshold point is lowered for the reason that sugar in the urine appears with a normal plasma glucose level.

As Smith¹ states, “The nature of the glucose ‘threshold’ mechanism of the kidney can be examined by the simultaneous determination of the rate of glucose filtration and the glucose clearance. The quantity of glucose filtered per minute will be the filtration clearance (in the dog, the creatinine clearance) multiplied by the concentration of glucose in the plasma; the difference between this quantity and the quantity excreted per minute gives the quantity reabsorbed by the tubules. The glucose clearance is essentially zero until the plasma level reaches a critical value (the ‘threshold’); above this level glucose begins to be excreted, the clearance increasing rapidly with rising plasma level and approaching the filtration rate as its asymptote. Ni and Rehberg,² assuming that reabsorption takes place in the proximal tubule before any reabsorption of water occurs, calculated the equilibrium concentrations in the blood and urine at various levels of hyperglycemia and concluded that the reabsorptive process is limited by a maximal concentration difference between these two fluids. They recognized, however, that the quantity of glucose which the tubule cells could reabsorb per minute might have some upper limit. But in point of fact, so long as the rate of filtration remains constant, the reabsorption of a constant quantity of glucose will lead to a constant concentration difference between tubular urine and blood, under their assumption, so that this latter relationship may be purely fortuitous.”

Glucose is almost completely reabsorbed by the tubules when it is present in the glomerular filtrate in the same concentration as in blood plasma. If, however, the plasma level is elevated, part of the glucose is not reabsorbed and escapes into the urine.

The transit of urea from the blood into the urine seems to occur in the glomerulus. There is no evidence at this time that there is any active reabsorption of urea in the sense that glucose is reabsorbed by the tubules. A test for glomerular function consequently must be the urea clearance test, which is fully explained on page 261 of this text. Urea clearance means the volume of

¹Smith, H. W.: *The Physiology of the Kidney*, pp. 111-112, London, 1937. Oxford University Press.

²Ni, Tsang-G., and Rehberg, P. B.: *Biochem. J.* 24: 1039, 1930.

blood cleared of urea by one minute's excretion of urine. An adult with a normal volume of urine excretes per minute the amount of urea in 75 c.c. of blood. According to Smith, "the deficit in the urea clearance is due to the escape of urea from the lumen of the tubules back into the blood in consequence of a high concentration gradient established by the reabsorption of water, supplemented by an as yet unknown reabsorptive process which takes up something like 40 per cent of the filtered urea at the highest urine flow obtainable. During the time when the urine flow is increasing, the urea clearance may be raised to abnormally high values, relative to the rate of urine formation. In view of the multiplicity of the factors influencing the rate of excretion of urea, it appears best to adhere to the empirical description of this process in terms of standard and maximum clearances, using the latter wherever practicable."

Another method is the **Ambard coefficient**. In this test the rate of urea excretion in the urine varies directly as the square of the urea concentration in the blood. With urea concentration of the blood remaining constant while that of the urine varies, the rate of urea excretion of the urine is inversely proportionate to the square root of its concentration in the urine. Furthermore, if both the blood urea concentration and the concentration of urea in the urine are variable, the rate of urea excretion varies directly as the square of urea concentration in the blood and inversely as the square of urea concentration in the urine. Ambard¹ has given us his formulae as follows:

$$\frac{\text{Urea in Blood}}{\text{Rate of Excretion}} = \text{Constant, or } \frac{\sqrt{\text{Urea in Blood}}}{\sqrt{\text{Excretion per unit of time}}} = \text{Constant.}$$

Again, the

$$\frac{\text{Rate of Excretion I}}{\text{Rate of Excretion II}} = \frac{\sqrt{\text{Concentration II}}}{\sqrt{\text{Concentration I}}}$$

Expressed by Mosenthal and Lewis,² the formula is as follows:

$$K = \frac{Ur}{\sqrt{D \times \frac{70}{P} \times \frac{C}{25}}}$$

In which K = the coefficient of urea excretion.

Ur = urea grams per liter of blood.

D = urea grams excreted in urine in 24 hours.

C = urea grams per liter of urine.

P = body weight in kilograms.

70 = standard body weight in kilograms

25 = standard concentration of urea grams per liter of urine.

K, or the coefficient of urea excretion, lies between 0.06 and 0.09 for a body weight of 70 kilograms with an average of 0.08.

The constant is unaffected by diet. With increasing degrees of renal impairment the constant rises. A constant above 0.09 indicates impairment of urea excretion; in fact, in severe grades of nephritis, values of 0.30 to even 1.0 may be encountered. Quoting from Mosenthal and Lewis,² "When the values rise above 0.09, some impairment of the power of the kidney to excrete urea is in-

¹Ambard: *Physiologie normale et pathologique des reins*, Paris, 1914.

²Mosenthal and Lewis: *J. A. M. A.* 67: 933, 1916.

dicated. Inability of the kidney to eliminate urea in proportion to the concentration of the blood urea results in an increase in Ambard's coefficient. In a normal individual it will remain within the limits mentioned, no matter what the height of blood urea; in persons with impaired renal function, however, the kidney does not answer the diuretic stimulus of the blood urea adequately, too little urea is put out, and the result is a rising coefficient, whether the urea in the blood be high or low. This test of Ambard is important in the detection of early kidney functional deficiency.

Van Slyke and Cullen* conclude as follows regarding the Ambard coefficient:

1. The Ambard formula (original or modified by McLean) is not a precise expression of the law of renal function insofar as elimination of urea is concerned. This is particularly true as regards the effect of urinary urea concentration.

2. The upper limit of blood urea in normal individuals taken under normal circumstances is 0.35 gm. urea per liter of blood. Higher figures, under normal conditions, indicate evidence of impaired renal function.

3. With McLean's modification of Ambard's formula, in the majority of nephritic cases, a lowering of the index is accompanied by a rise of blood urea above the 0.35 gm. per liter limit. The index affords no valuable diagnostic or prognostic information which one cannot deduce from the blood urea alone.

4. In some instances, for example, arteriosclerosis and cardiac decompensation, blood urea was normal with a lowered index. Since it is important to distinguish between vascular and renal cases, the index loses its clinical value.

5. There is possibility of error from undetected incomplete collection of the urine. This cannot occur in a simple blood urea estimation.

6. Urea index estimations in the same individual yield wider variations in normal or nonnephritic individuals than in nephritics.

7. The estimation of blood urea is more useful for ordinary clinical diagnosis and prognosis than the estimation of the urea index or the Ambard quotient.

Usefulness of Blood Chemical Tests.—It is true that there may be severe impairment of kidney function with a normal blood chemistry because the patient has so adjusted his food intake as not to exceed the functional capacity of the kidney or because the diminished concentrating capacity of the kidney has been balanced by a relative polyuria. For the estimation of the impairment of renal function, particularly so far as the glomerular function is concerned, blood chemical methods alone constitute the laboratory criterion of accumulation of the urinary constituents in the blood. The substance in which we are the most interested is the accumulation of urea. The accumulation of total nonprotein nitrogen is not nearly so important in this regard as is the accumulation of urea because there is much more nonprotein nitrogen in the tissues than in the blood. The estimation of the other nonprotein nitrogenous substances, such as uric acid, creatinine, indican, etc., while important, may be routinely dispensed with in the estimation of kidney function. The interpretation of the urea content of the blood must be guided somewhat by the previous diet of the patient. This was well illustrated by von Dollivo.¹ He found that in the normal person two hours after the ingestion of 20 gm. of urea the urea content of the blood rises to about 40 mg. per cent above the initial value.

*Van Slyke and Cullen: *J. Biol. Chem.* 19: 211, 1914.

¹Von Dollivo: *Deutsches Arch. f. klin. Med.* 131: 109, 1919-1920.

It then declines, to reach the normal in somewhat less than twenty-four hours. He found that patients with nitrogen retention reach a maximum more slowly, attaining it in about four to six hours, the increase in urea concentration being 60 to 70 mg. per cent. In addition to this, patients with renal insufficiency require two to three days to return to the original values. King² obtained similar results, according to studies he made on the effect of the ingestion of urea on the level of this substance in the blood.

It is evident, therefore, that, while making chemical tests of blood, data on the previous diet of the patient must be known. As Fishberg³ states: "This is also illustrated in patients with the oliguria of chronic nephrosis who may have considerable elevation of the nonprotein nitrogen of the blood as a result of high protein diet and the administration of urea, even though renal function is unimpaired. The prognostic significance of nitrogen retention in one who has previously been on rigorous protein restriction is more serious than in one who has been eating meat and other nitrogenous foods at will."

We know that urea nitrogen accumulates in the blood in renal insufficiency. The normal value is about 10 to 15 mg. per 100 c.c. of blood. This may rise to as much as 500 mg. Uric acid usually parallels urea, but exceptionally it may rise before urea begins to accumulate. Fishberg found hypertensive patients with elevated blood uric acid for protracted periods which did not indicate the onset of renal insufficiency. In general, it may be stated that uric acid accumulation, indicative of kidney functional impairment, goes hand in hand with blood urea accumulation.

Creatinine, according to the observation of Myers, Fine, and Lough,⁴ is the last substance to accumulate in the blood following derangement of kidney function. It is therefore to be found in large quantities in the terminal stage of kidney function impairment.

Amino Acids.—There is generally no elevation of amino acids and other protein decomposition products under circumstances of kidney failure. Bennett⁵ found amino content of the blood high in the terminal stages of uremia. According to Rabinowitch,* there is no increase in ammonia as a result of impaired kidney function. According to Nash and Benedict,† the ammonia found in the kidney has its origin in the kidney, which explains the depletion of the alkali reserve in the body under certain conditions. If the efficiency of the kidney becomes impaired, the formation of ammonia is diminished or if it does not keep pace with the rate of an excessive acid production, other fixed bases in the body must be called upon in the regulation of the body neutrality. In other words, in nephritis we have here an explanation of the increase in the intensity of the urinary acidity. It is to the study of urinary acidity that we must turn in our estimation of renal efficiency rather than to the study of data determined from

*Rabinowitch: *Canad. M. A. J.* 8: 742, 1923.

†Nash, T. P., and Benedict, S. R.: *J. Biol. Chem.* 48: 463, 1921.

²King: *Arch. Int. Med.* 42: 877, 1928.

³Fishberg, Arthur M.: *Hypertension and Nephritis*, ed. 3, p. 64, Philadelphia, 1934, Lea & Febiger.

⁴Myers, Fine, and Lough: *Arch. Int. Med.* 17: 570, 1916.

⁵Bennett: *Lancet* 1: 535, 1928.

an analysis of ammonia findings in the blood under these circumstances. The ammonia of urine, in other words, may be an index of impaired renal efficiency but it is no index as to the degree of acid production. It is generally believed that the kidney is the seat of urinary ammonia formation.

Chlorides.—There is usually a diminution in the amount of chlorides in blood in cases of impaired kidney function. This is due to loss of chlorides by vomiting and ingestion of little or no salt. De Wesselow¹ found in advanced nephritis an inability of the kidney to excrete phosphoric acid, with resulting accumulation of the acid radical in the blood. Alterations in the inorganic blood chemical picture of the nephritic in the terminal stages of the disease are (1) a great increase in the phosphoric and sulphuric acid radicals, and (2) since acids circulating in the blood are of necessity neutralized, abstraction of base from the plasma bicarbonate for combination with the acid excess occurs and the resulting volatile carbonic acid is excreted by the lungs. Though the diminution in the bicarbonate of the plasma is reasonably ascribed to the accumulation of phosphoric acid in the blood, it has been pointed out by Denis and Minot² that the phosphate increase and the bicarbonate decrease are not in all cases proportional. It is probable, according to de Wesselow, that some form of redistribution of base must occur, in which acid radicals, other than the bicarbonate and phosphate, play a part. In an observation on one case, de Wesselow noted that while the phosphate and the various basic radicals remain comparatively constant, the bicarbonate and chloride varied inversely. The rise in the bicarbonate content is accompanied by a fall in the chlorides.

This, therefore, could be interpreted as a result of the shift of hydrochloric acid to the corpuscles. Blood was withdrawn from this patient at the end of the period of continuous uremic vomiting. It was already known that in the vomiting of intestinal obstruction an extreme fall in the chloride content of the blood occurs and that at the same time the plasma bicarbonate rises to a figure much above the upper limit of the normal. These changes occur probably in the following way: Large quantities of hydrochloric acid are lost in the vomitus; this hydrochloric acid is derived in the process of gastric secretion from the sodium chloride of the plasma, the residual sodium combining with carbonic acid in the blood to form bicarbonate. Under normal conditions the excess bicarbonate thus formed passes into the intestines with the pancreatic and intestinal secretions, and combines with the gastric hydrochloric acid to form sodium chloride, which can be reabsorbed. With vomiting the circle is broken, and, while the chloride content of the blood falls, the bicarbonate content rises.

It would appear then that the vomiting of uremia may be regarded as constituting a protective mechanism. By removal of hydrochloric acid, base is provided and the bicarbonate content is to some extent conserved in spite of phosphate retention. The vomiting is not the result of the reduction of the bicarbonate, since vomiting is by no means a marked feature of diabetic acidosis.

For a more complete discussion of chlorides, refer to pages 395 ff. and 429.

¹De Wesselow: *Lancet* 1: 1099, 1924.

²Denis and Minot: *Arch. Int. Med.* 26: 99, 1920.

Phosphate.—Greenwald¹ found an increase in inorganic phosphate as a result of renal insufficiency. As much as 10 mg. per 100 c.c. are not uncommon in the plasma of uremic patients. The normal in adults is about 3 mg. per 100 c.c. This accumulation is due to the changes in the lecithin content of the blood that accompany alterations in the level of the plasma proteins. De Wesselow has published some interesting observations on the high concentrations of phosphates seen in uremic patients.

Sulphates.—It is claimed by many authorities that inorganic sulphates may accumulate in the blood in renal insufficiency, among others, Denis and Hobson.² These two workers, in a study of twenty-two cases of nephritis and cardiorenal disease, found there was a marked increase over normal of the sodium and chlorine in only four cases; the inorganic phosphate fraction was increased in ten cases; while the inorganic sulphates were determined in only seventeen cases, eleven of which showed increased values. Magnesium and potassium remained more or less constant while calcium was found to be decreased in five cases. Their studies demonstrated the specific selective activity of the kidney towards the various normal inorganic constituents of serum, somewhat similar to the selective activity which has been shown to exist for the various nonprotein constituents of blood.

It is probable that sodium and chlorine, like creatinine, are excreted with great ease and, even in the case of badly damaged kidneys, retention of these elements seldom occurs. On the other hand, the sulphate ion is apparently excreted with difficulty so that in kidney insufficiency the concentration of this fraction may increase enormously, in some cases to 3,000 per cent above the normal value; in this respect the sulphate ion may be compared with the uric acid fraction although the percentage increases observed are far above any concentrations of uric acid so far observed.

The Relationship Between Uremia and Blood Chemical Findings.—It is generally believed that uremia is the direct result of accumulation of urea and kindred substances in the blood. Bright is responsible for this theory. In his Goulstonian lecture, delivered in 1833, he said: "What seems in some way to account for the general derangement and suffering of the constitution is the fact that urea becomes demonstrable in the circulating mass, and the blood becomes impregnated with that substance." Over one hundred years after this statement was made, this conception of the significance of urea retention has been amplified. We know now that urea is but one of the nitrogenous bodies which accumulate in the blood in nephritis and have learned that a similar retention occurs in the case of certain nonnitrogenous substances. This aspect of nephritis was given a fresh impetus at the beginning of the present century by the work of Widal and Javal and by that of Achard. Bennett³ in his first Goulstonian lecture in 1928 in discussing "Some Problems of Nephritis" states that while the retention of nitrogenous substances in the blood is an invaluable help in the diagnosis of renal failure, and though one may appreciate the fact that high nitrogen figures may become of great prognostic importance, one must also

¹Greenwald: *J. Biol. Chem.* 21: 29, 1915.

²Denis and Hobson: *J. Biol. Chem.* 55: 183, 1923.

³Bennett, T. I.: *Lancet* 1: 535, 1928.

realize that nitrogen retention by itself offers no explanation of the actual phenomena of uremia. In summarizing the chief reasons against nitrogen retention as the cause of uremia, Bennett gives the following:

“1. The administration of urea and kindred substances to animals does not produce uremia.

“2. Conditions such as cystic disease of the kidney or prostatic obstruction often persist for years with marked nitrogen retention but no evidence of uremia.

“3. Cases of uremia without nitrogen retention are occasionally observed.”

The retention of nitrogenous substances in the blood is marked in renal failure, according to Bennett, largely because they are quantitatively among the more important substances which the kidney is normally called upon to excrete; but evidence accumulates to show that other chemical substances will show increased concentration in the blood, although to a lesser degree. In a very interesting case of cystic disease of the kidneys which Bennett was enabled to study up to the very last moment of life (ending in uremia) the blood analyses were in full agreement with the findings of most modern authorities, actual figures in the terminal stage being as follows:

	BLOOD PHOSPHORUS (NORMAL, 2-3)	BLOOD CHLORIDES (NORMAL, ABOUT 500)
Nov. 2	6.3	503
Nov. 4	6.2	561
Nov. 5	8.3	568
Nov. 6	8.5	553

Bennett calls attention to the curious diazo reaction, first described by Andrews, given by the blood and cerebrospinal fluid in cases of uremia. It has been shown by Blotner and Fitz¹ to appear rapidly in dogs after nephrectomy, and, though its significance is still obscure, it seems very possible that this again is an example of the retention and concentration in the blood of some substance which the normal kidney would excrete.

Bennett in his lecture called attention to the outstanding features of similarity in cases of advanced renal destruction and cases of pyloric obstruction with tetany. He showed that in progressive renal disease there is found the following:

- Acid accumulation in blood.
- Acidosis.
- Phosphorus retention.
- Nitrogen retention.
- Calcium reduction.
- Twitchings and convulsions.

He found the following in nonmalignant pyloric obstruction:

- Acid loss from vomiting.
- Alkalosis.
- Chloride loss.

¹Blotner, H., and Fitz, R.: J. A. M. A. 88: 985, 1927.

Nitrogen retention.

Calcium reduction.

Tetany.

He called attention to the literature in which cases of pyloric or upper intestinal obstruction, in which routine histologic examination of kidneys removed at autopsy showed entirely unsuspected changes of a markedly degenerative nature in the renal tubules, were described. These changes were of the type seen in cases of mercurial poisoning. Crohn¹ reported a case of a patient who had undergone an operation for pyloric obstruction in whom uremia, resulting from this renal complication, was the cause of death. It is evident that not only will renal damage produce profound changes in the blood chemistry but that chemical changes in the blood may themselves exert a destructive effect upon the kidney substance.

Bennett has given full credit to the information obtained from biological chemists in elucidating facts concerning abnormal physiology concerning the kidney, but the fact remains that the phenomena of uremia are not altogether explained by the accumulation of blood chemical products in kidney degeneration.

Tests for Kidney Function

There are a number of tests of kidney function, each group dependent upon certain physiologic principles. According to Belt,² the requirements of a test of renal function may be summed up as follows:

1. It should be easy for the physician to apply and for the patient to carry out.
2. It should be easy of interpretation.
3. It should be carried out through the agency of a nondeleterious substance, preferably one with which the kidney is regularly called upon to deal.
4. The substance used should be one which is not influenced by any other organ in the body.
5. The test should reveal slight impairment and yield an answer in per cent of normal, ranging uniformly through all changes, from slight impairment of function and structure to a total loss of kidney function.
6. It should reveal the nature of anatomic change occurring within the kidney.
7. The test applied in a chronologic series should have a prognostic value, indicating further degeneration or recovery.

Belt states further that no single test encompasses all of these criteria, that while the urea clearance test is constant, exact, and covers the greatest range of function, it has the disadvantage of requiring technical aid in the form of chemical estimates of urea in both blood and urine. The range of the phenolsulphonephthalein test is much more restricted, being insensitive to slight failure in function and diminishing to the zero point long before the kidneys have approached a level of function in which life is in danger. As

¹Crohn, B. B.: *Affections of the Stomach*, p. 660, London, 1927, W. B. Saunders Company.

²Belt, E.: *Modern Tests of Renal Function*, Cabot's "Modern Urology," Vol. II, 1936, Lea & Febiger, Philadelphia, Pa.

pointed out by Frothingham and Dennis, it indicates the function for the moment and not over an extended period of time. Its greatest value, which accounts for its popularity, is the fact that the laboratory procedures involved require the minimum of effort.

Dilution and concentration tests give the earliest warning of an impending failure of renal structure. Belt further emphasizes that the qualitative tests for albumin in the urine are very delicate and reveal quantities of this substance smaller than are pathologically significant. The xanthoproteic test gives a warning highly significant for judging danger to life.

Urea Clearance Test.—For full details of the technic, see pages 262 to 265. This test depends upon the excretion of a natural metabolite as it occurs in the body without artificial administration. For full data on the significance on the urea clearance figures, see pages 176 and 261. In general, a reading of 5 per cent shows that uremia is near at hand. The patient is still able to be up and about with readings of 10 per cent. Twenty per cent is recognized as a dangerously low level though at this level an existing edema may clear and a sense of improvement in physical welfare be noted by the patient. At 52 to 56 per cent the phenolsulphonaphthalein test begins to be impaired.* Readings of 70 per cent or above indicate normal function. High readings indicate hyperfunction.

Sources of error in this test occur through inaccuracy in timing urine collections and incomplete emptying of the bladder.

The great value of this test is demonstrated in two charts reproduced from Van Slyke.†

In explaining these charts, Van Slyke stated that in Case 1 (Fig. 73) the clearance was first noted one month after onset, at 15 per cent of average normal. Then during two months it rose to 100 per cent. The specific gravity, which was very low at 1.010, showed no rise at all during these two months. Improvement in this patient was genuine and was followed by complete recovery. The specific gravity figures six months after discharge were also found to be normal. For some time after the second month an abnormality remained in the kidneys which, although it did not interfere with urea excretion, was nevertheless indicated by the low specific gravity values which the concentration test continued to yield.

In Case 2 (Fig. 74) during the first seven months the specific gravity test followed the urea clearance more or less closely. However, between the seventh and tenth months the specific gravity fell to 1.010 which is nearly the minimum reached in terminal nephritis. At this time, the urea clearance still showed 30 per cent of normal function. During the subsequent twenty months, while the clearance gradually approached the uremia level at 5 per cent of normal, the specific gravity showed no further fall.

The Dye Test of Renal Function.—A number of different substances have been suggested for the so-called dye test of kidney function. The first dye used was methylene blue, which was introduced by Achard and Castaigne^{1, 2}

*Harding, V. J., and Urquhart, V. J.: *J. Urol.* 29: 1, 1933.

†Van Slyke, D. D.: *M. Clin. North America*, pp. 1179-1193, March, 1934.

¹Achard, Ch., and Castaigne, J.: *Diagnostic de la perméabilité rénale. Bull. et mém. Soc. méd. d'hôp. de Paris* 14: 637, 1897.

²Idem: *Diagnostic de la perméabilité rénale, Gaz. hebd. de méd. Paris* 37: 433, 1897.

in 1897. Later, indigo carmine was used by Heidenhain¹ in his investigation of the physiology of the kidney. Later on, in 1903, Voelker and Joseph suggested the use of the dye for the testing of renal function. Twenty c.c. of a 0.4 per cent solution were injected into the muscles of the gluteal region. Normally, the urine became greenish blue from ten to fifteen minutes after the injection. Our experience with indigo carmine has been limited to the separate determination of the functions of the kidney when a cystoscope is introduced into the bladder, and the appearance of colored urine trickling down

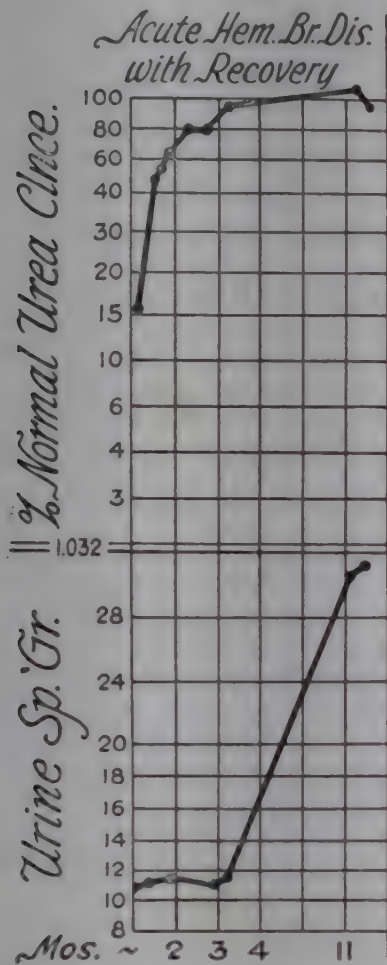


Fig. 73.

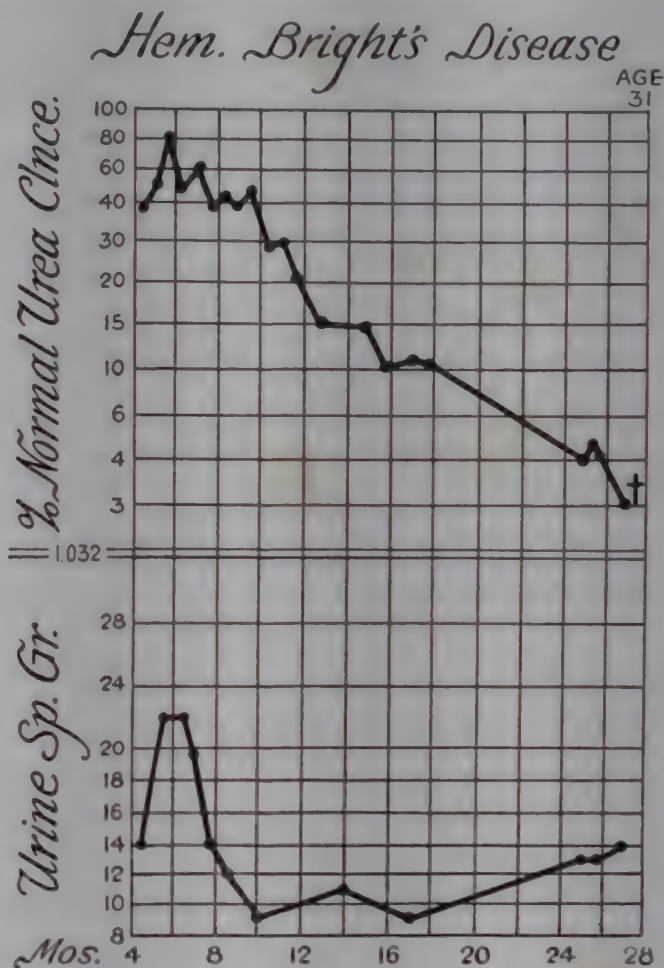


Fig. 74.

Fig. 73.—Recovering case of acute hemorrhagic nephritis. Changes of urea clearance and of urine specific gravity in Addis concentration test. Note that three months after onset the clearance had returned to full normal value, while specific gravity test showed no improvement. Eleven months after onset both tests normal. (Alving and Van Slyke, unpublished.)

Fig. 74.—Case of acute hemorrhagic nephritis with temporary improvement, followed by lapse into progressing chronic nephritis with eventual death in uremia. (Alving and Van Slyke, unpublished.)

from each side noted. This was termed chromocystoscopy. Rosaniline was introduced by Lepine* in 1898. One cubic centimeter of a 1 per cent solution was injected subcutaneously. Geraghty and Rowntree² deserve credit for introducing the phenolsulphonephthalein test for estimating renal function.

The phenolsulphonephthalein test is frequently used in medical and surgical diagnoses. It has a definite field of usefulness but cannot be relied upon to the exclusion of other methods of testing kidney function. A low phenolsulphonephthalein output is very suggestive of renal injury. In surgical cases, in connection with operability or operative risk, the test is quite useful.

*Lepine: Sur la perméabilité rénale, Lyon Méd. 87: 251, 1898.

¹Heidenhain, R.: Herman's Handbuch der Physiologie 5: 348, 1883.

²Geraghty, J. T., and Rowntree, L. G.: J. A. M. A. 57: 811, 1911.

Phenolsulphonephthalein Test of Kidney Function.—

Reagents.—

Ampules of Phenolsulphonephthalein may be obtained from Hynson, Westcott, and Dunning, or prepared as follows:

Dissolve 0.6 gram phenolsulphonephthalein
in 0.84 c.c. 2/N sodium hydroxide in 0.75% sodium chloride solution.
Dilute to 100.0 c.c. with 0.75% sodium chloride solution.

This is the monosodium or acid salt, which is slightly irritant locally when injected.
Add 2 drops of 2/N sodium hydroxide.

Solution should change to a Bordeaux red. This solution is nonirritant.

Ampule in 1.5 c.c. quantities, seal hermetically, and sterilize.

One c.c. of this dye is injected intramuscularly.

Standard Solution of Phenolsulphonephthalein. (100 c.c. contains 0.6 mg. phenolsulphonephthalein.)

Dissolve 1 c.c. phenolsulphonephthalein solution (above)
in 10 c.c. 10% sodium hydroxide and
Dilute to 1000 c.c. with distilled water.
The solution may be kept one month.

10% Sodium Hydroxide.

Dissolve 10 gm. NaOH in enough distilled water to make 100 c.c. Keep tightly stoppered.

Technic.—

Instruct the patient to omit breakfast when he reports to the laboratory in the morning.

Give the patient 300 to 400 c.c. water about one-half hour prior to the injection.

Half an hour later, instruct him to urinate and discard the specimen.

Inject 1 c.c. of the phenolsulphonephthalein solution intramuscularly in the lumbar region, or, preferably, intravenously.

Allow ten minutes for the beginning of the excretion of the drug.

One hour and ten minutes after the injection, collect the urine in a bottle labeled "No. 1." Collect the entire specimen.

Give the patient 300 c.c. water.

One hour after this, instruct the patient to collect the second specimen in a bottle labeled "No. 2." In patients with obstruction to the flow of urine, the urine is drawn off at the end of each hour. Other patients may simply be allowed to urinate at hourly periods.

To the entire first-hour specimen in bottle No. 1, add 10 c.c. 10% sodium hydroxide.

Rinse the mixture into a 1000 c.c. graduated cylinder with a few c.c. of water.

Dilute to 500, 750, or 1000 c.c. depending on the depth of color. The color must be lighter than the color of the standard solution.

Read in the colorimeter.

Calculate the results of the first specimen.

Carry out the same procedure with the second specimen, and calculate the results. Report the entire amount of dye excreted.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{\text{Dilution of Unknown}}{\text{Dilution of Standard}} = \text{the \% of dye excreted.}$$

Example.—

Reading of standard is 15; reading of unknown is 20; dilution of standard is 1000; dilution of unknown is 750.

$$\frac{15}{20} \times \frac{750}{1000} = 56\frac{1}{4}\%.$$

Suppose 56¼% of dye were eliminated the first hour, and 30% the second hour. Report as follows:

First hour -----	56¼%
Second hour -----	30 %
Total dye eliminated -----	86¼%

Normally about 75% of the dye should be excreted in 2 hours.

This is a very important test in connection with the estimation of kidney function. Normal kidneys excrete from 60 to 75 per cent of this dye in the two-hour period allowed for the test. A fall to 40 per cent indicates impaired function. Reduction to 20 or 30 per cent is the rule in well-marked cases of nephritis, while excretions of less than 10 per cent may be interpreted as very serious. The exact interpretation of the findings of the phenolsulphonephthalein test, however, indicates a pathologic condition of the kidneys for the moment and not over an extended period of time. In order to obtain a true estimate of the condition of the kidneys over an extended period of time, one must resort to blood chemical methods.

According to the work of Frothingham and Dennis, it is known that the phenolsulphonephthalein test may be normal or may be increased in acute glomerulonephritis. In certain other cases of acute nephritis, there may be an extremely low excretion, almost to zero. These figures are based largely upon experimentally induced nephritis, induced by the injection of uranium nitrate. In chronic nephritis in clinical practice we have known the phenolsulphonephthalein output to be between 70 and 85 and still there was marked damage to kidney function. It is a clinical fact that low excretion from 10 to 15 per cent is the rule in cardiac decompensation with chronic passive congestion of the kidneys. This rises rapidly when the congestion is relieved by improved heart action. In chronic nephritis, although the estimation of this test is important, it sometimes presents inexplicable variations.

LaMotte Phenolsulphonephthalein Apparatus Roulette Type

Phenolsulphonephthalein is injected and the specimen collected as in the test described on page 164. The method of reading the test, in which advantage is taken of the chemical indicator properties of phenolsulphonephthalein, may be termed the bicolor method. This completely eliminates the effects of color and turbidity of the specimen in making the color comparison.

Concentration and Dilution Tests

As indicated before, the concentration tests of urine are mainly for the purpose of determining the functional ability of the tubules.

The Two-Hour Specific Gravity Test of Mosenthal.—

This is a simple method of ascertaining the concentration power of the kidneys. The specific gravity is taken on two-hour samples of day urine, and the amount of the night urine is then measured. For healthy individuals this amount is 400 c.c. or less, while the specific gravity is 1.018 or higher. In the two-hour samples of the day urine, the specific gravity fluctuates ten points or more from the highest to the lowest point.

Impairment of kidney function is noted by an increase in the night urine, that is, it goes above 400 c.c., while the specific gravity is low, around 1.015. Lower specific gravity is seen with very little variation in the two-hour specimens of day urine. The specific gravity is fixed and does not change.

The patient must be directed not to take any liquids or foods after supper or in the morning until the morning urine is voided. He is permitted to take breakfast or noonday meal of an ordinary type, but must take no liquids or foods between meals.

This test is extremely valuable in nephritis because it tells some general points about kidney efficiency and gives data on the water excretion. According to Wohl¹ the test is characteristic in myocardial decompensation. There is oliguria with a markedly fixed specific gravity of about 1.020. In the period between cardiac decompensation and the beginning disappearance of the edema, specific gravity is low and fixed, and a nocturnal polyuria exists. For a simple test of the functional reserve power of the kidney, give the patient for one day a diet of 20 per cent solid food only, with no fluids. Collect the urine at three-hour intervals. In normal urines the specific gravity usually reaches 1.030.

Interference with the transudative power of the glomeruli and the secretory property of the tubules can be ascertained by observing the disproportion between fluid intake and fluid elimination. Use the **water test of Volhard**.

Patient must remain in bed during the test. Give 1500 c.c. water in the morning on an empty stomach. Collect the urine every hour for four hours, and later every two hours. After four hours, the patient is on a dry diet. Normally most of the consumed liquid is eliminated during the four hours; small amounts are eliminated during the latter part of the day. In the second and third hours after water consumption, specific gravity varies between 1.001 and 1.003, rising later in the day to 1.027.

Deviations from the normal: If, in the first four or five hours, more urine is eliminated than water consumed, e.g., 1,600 c.c., with a specific gravity of about 1.008 to 1.010, but not very low (isothermuria), it indicates severe kidney damage; this occurs in nephrosclerosis (arteriolar nephritis) or when edema fluid is absorbed. Some patients may eliminate less fluid than was consumed, as in cases approaching edema, such as hypothyroidism, cardiac decompensation, and in the later stages of nephrosis.

The Fishberg Concentration Test.—

This is a simpler method than that of Mosenthal. It is fully described in the classic volume of A. M. Fishberg, *Hypertension and Nephritis*. On the evening before the test, the patient takes his supper at 6 P.M. This meal should contain a large amount of protein, but the fluid intake should not be more than 200 c.c. No food or drink may be taken after this until the test is finished. The urine should be voided before retiring and should be discarded. The first specimen saved is the morning urine. The patient remains in bed and another specimen is collected one hour after the first in another container. A third specimen is collected in still another container two hours after the

¹Wohl, M. G.: *Bedside Interpretation of Laboratory Findings*, pp. 201, 202, St. Louis, 1931, The C. V. Mosby Company.

first. The specific gravity of each of the three specimens is taken. Under normal conditions, the specific gravity should be over 1.024 in at least one specimen.

This test is based upon the fact that concentration is the most essential function of the normal kidney, especially the tubular portions, and that any decrease in this quality is a prime indication of renal impairment. Accordingly, under the conditions of this test the specific gravity, which indicates the concentration of urinary constituents, should be increased if the kidney function is normal, for the reason that no fluid is being ingested during the performance of the test. At the same time, if the kidney function is materially disturbed, the specific gravity will be lower than usual. Accordingly, the figure 1.024 is fixed as the minimum level for normal urine. In cases of uremia, the specific gravity figures are surprisingly lower, sometimes as much as 1.010.

The test cannot be used when there are conditions of uremia, but it is very useful in early cases of renal impairment, especially to indicate the impairment of the tubular portions of the kidney.

The Sodeman-Engelhardt Concentration Test.—*, 1-3

The renal concentration test employing posterior pituitary extract is based upon the renal action of this substance, which has been shown to inhibit water diuresis as well as increase the chloride excretion. It reduces urinary volume and increases the concentration of solids in the urine. This action takes place in spite of the ingestion of water, so that previous preparation of the patient by fluid restriction is unnecessary.

In practice the following procedure is carried out:

1. The unprepared subject empties the bladder and the specimen is saved.
2. Immediately 10 units (1.0 c.c. obstetrical or 0.5 c.c. surgical) posterior pituitary extract is given subcutaneously.
3. The subject receives nothing by mouth until the test is completed.
4. Specimens are obtained at the end of the first and second hours.
5. The specific gravities of the three specimens are then taken, and the usual corrections for albumin and temperature are made, if necessary.

In normal individuals at least one of the specimens will show a specific gravity of 1.022 or more. Urinary volumes may be small, but cheap and accurate urinometers requiring only 4 to 6 c.c. of urine are available.

The dosage of posterior pituitary extract used does not affect the blood pressure. Hypertension is not a contraindication to the test. The extract causes coronary constriction and should be used with caution in angina pectoris. Acute coronary occlusion, sensitization, and late pregnancy are contraindications to its use.

The test is simple, requires only minimal cooperation by the patient, particularly as far as fluid restriction is concerned, and can be carried out imme-

*Courtesy of the originators of the test, Drs. W. A. Sodeman and H. T. Engelhardt of New Orleans, La.

¹Sodeman, W. A., and Engelhardt, H. T.: (A) *Proc. Soc. Exper. Biol. & Med.* 46: 688, 1941; (B) *Am. J. M. Sc.* 203: 812, 1942.

²Talbot, J. H.: *New England J. Med.* 226: 197, 1942.

³Pasqualini, R. Q., and Etala, E.: *Rev. Soc. Argent. de Biol.* 16: 161, 1940.

diately, without preparation of the patient, at any time of day. It is particularly indicated in edematous patients where other concentration tests are inaccurate because of diuresis. Furthermore, it is of value in surgical patients where restriction of fluids is contraindicated.

Concentration and Dilution Tests of Volhard and Fahr.*—

1. Concentration Procedure.—(a) Allow no fluids from the evening before the test until the test is completed and no food between meals. All three meals must be without liquids and without liquid food.

(b) 8:00 A.M. Patient empties bladder. Thereafter collect urine in separate containers every three hours until 8:00 P.M.; that is, at 11:00 A.M., 2:00 P.M., 5:00 P.M., and 8:00 P.M., and collect all urine from 8:00 P.M. until 8:00 A.M. the next morning in one container.

(c) Note the quantity and specific gravity of each three-hour sample and of the twelve-hour sample and plot as a curve. Normally, the specific gravity of at least one sample should be 1.030 or at least 1.025.

2. Dilution Procedure.—(a) Omit breakfast on the day of the test. For lunch and dinner give the patient the diet to which he has been accustomed.

(b) 8:00 A.M. Patient empties bladder and is given 1500 c.c. water.

(c) Collect urine in separate containers at 8:30, 9:00, 9:30, 10:00, 10:30, 11:00, 11:30 A.M., and 12:00 noon, eight specimens in all.

(d) Collect all the urine from 12:00 midnight to 8:00 A.M. the next morning in one container.

(e) Note the quantity and specific gravity of each sample and plot as a curve. Normally, the total quantity voided should be 80 to 120 per cent of the intake (1200 to 1800 c.c.). The specific gravity of at least one sample should be as low as 1.003.

Isberg-Newburgh 18-Hour Concentration Test of Kidney Function.—

Isberg and Newburgh¹ described a simplified concentration test of kidney function based on the 38-hour concentration test of Lashmet and Newburgh.² The test is performed over a period of only 18 hours and does not require the use of a special diet. It is particularly convenient for use on ambulatory patients. A simple, rapid, and accurate method for quantitative determination of proteinuria may also be made. This test is based on the comparison of the turbidity of urine, in which protein has been precipitated by 2 per cent sulphosalicylic acid, with the turbidity of a permanent, standard suspension of inorganic substance.

Procedure.—The subject finishes his usual supper by 6 P.M. He then has nothing to eat or drink until noon the following day; he may carry on ordinary activity during this time. Collect urine specimens at 8 A.M., 10 A.M., and 12 noon, with complete emptying of the bladder each time. Determine the specific gravity of each specimen with the urine at room temperature and with an ordinary urinometer which has been checked with distilled water.

Test the most concentrated specimen for the presence of protein, and if present determine the amount quantitatively in order to correct the specific gravity when necessary. For every one per cent of protein present in the urine, subtract 0.003 from the observed specific gravity; any proteinuria less than 0.3 per cent does not need to be considered. Thus the specific gravity of most urines will not require correction.

Isberg and Newburgh have further simplified the easy and accurate method of Lashmet and Newburgh³ for quantitative measurement of proteinuria.

*Belt, E.: *Modern Tests of Renal Function*, Cabot's "Modern Urology," Vol. II, 1936, Lea & Febiger, Philadelphia, Pa.

¹Isberg, E. M., and Newburgh, L. H.: *Am. J. M. Sc.* 211: 6, 891, 701-704, 1946.

²Lashmet, F. H., and Newburgh, L. H.: *J. A. M. A.* 100: 1328, 1933.

³Lashmet, F. H., and Newburgh, L. H.: *J. A. M. A.* 100: 1328, 1933.

Reagents.—**Standard Turbidity Suspensions.—****Stock Suspension.—**

To 200 c.c. of distilled water in a 500 c.c. volumetric flask

add 50 c.c. of N/10 sodium hydroxide solution

, and 8 gm. of copper sulphate, hydrous.

Dilute to 500 c.c. with distilled water. Shake vigorously to insure uniform turbidity.

Standard Tube.⁴—

Shake the stock solution thoroughly and transfer exactly 2 c.c. to a test tube, using a pipette.

Add 23 c.c. distilled water. This standard tube can be made permanent by drawing out its open end and sealing in a flame. *Before using, invert the standard tube several times to obtain uniform turbidity.*

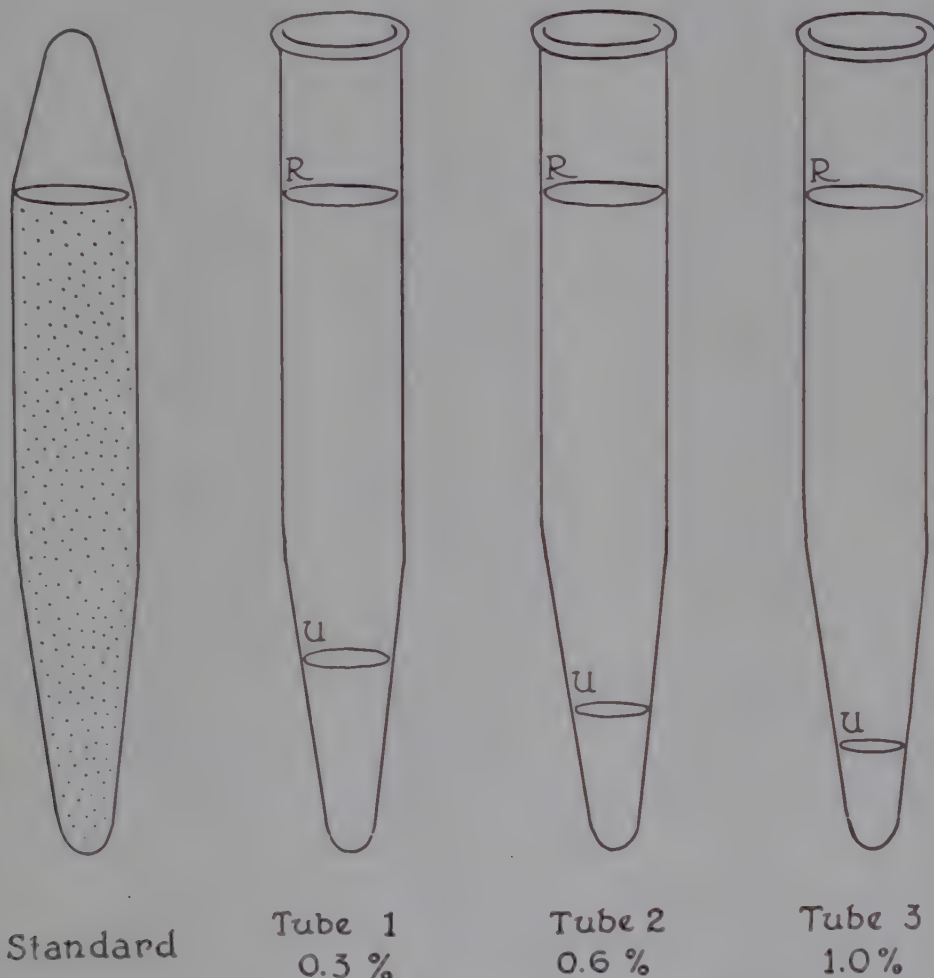


Fig. 75.—Set of tubes for rapid quantitative determination of proteinuria. *Tube 1:* U mark is at 1.66 c.c. = 0.3 % protein. *Tube 2:* U mark is at 0.83 c.c. = 0.6% protein. *Tube 3:* U mark is at 0.5 c.c. = 1.0 % protein. The R mark is at 12.5 c.c. (Courtesy Drs. Isberg and Newburgh: Am. J. M. Sc., June, 1946.)

2% Sulphosalicylic Acid.—

Dissolve 2 grams of sulphosalicylic acid in enough distilled water to make 100 c.c. solution. This reagent may turn pink on standing. Such solutions should be discarded, for the pink cast interferes with accurate comparison with the standard.

Technic.—

Use graduated 15 c.c. conical centrifuge tubes. Place the mark "U" at 1.66 c.c. for tube 1, at 0.83 c.c. for tube 2, and at 0.5 c.c. for tube 3. Place the mark "R" at 12.5 c.c. on each tube. Tube 1 represents 0.3% protein, tube 2, 0.6%, and tube 3, 1% protein. (See Fig. 75.)

⁴A set of calibrated tubes, standard tube, and a support (A 21-195) may be obtained from Eberbach and Son Co., Ann Arbor, Mich.

To 1 c.c. of urine, add 9 c.c. of distilled water, making a dilution of 1:10. Add the diluted urine to the U mark of tube 1 and then add 2 per cent sulphosalicylic acid up to the mark R. Mix thoroughly, and allow to stand for 3 minutes. If the resulting turbidity matches that of the *standard tube*, the urine contains 0.3% protein; the specific gravity of the urine is then corrected by subtracting 0.001. If the turbidity is less than that of the standard tube, no correction of the observed specific gravity is necessary.

If the turbidity of tube 1 is greater than that of the standard tube, add the urine diluted 1:10 to the U mark of tube 2, and then add 2 per cent sulphosalicylic acid to the R mark. Mix thoroughly and allow to stand; if the turbidity matches that of the standard tube, the urine contains 0.6 per cent protein. The observed specific gravity then is corrected by subtracting 0.002. If the turbidity is greater than that of the standard, add the diluted urine to the U mark of tube 3, and sulphosalicylic acid to the R mark. If the turbidity resulting in tube 3 matches that of the standard tube, the urine contains one per cent protein. The observed specific gravity is then corrected by subtracting 0.003. Urine containing more than one per cent protein should be diluted 1:20, and the foregoing procedure with the three tubes repeated.

A maximum specific gravity of 1.025 is considered the lower limit of normal for this 18-hour renal concentration test.

Of 200 cases of essential hypertension, 124 cases, or 62 per cent, showed impaired concentrating ability. The concentration test was found to be more sensitive than the urea clearance test in detecting early kidney damage in essential hypertension. These authors also found that in each of eight cases of glomerulonephritis, both subacute and chronic, there was impaired concentrating ability. The maximum specific gravity in these cases ranged from 1.010 to 1.020.

Addis Urea Concentration Test.*—

This test is for ambulant patients—not for cases with edema or very high diastolic pressure or failing heart or in the late stages of Bright's disease.

1. Give 20 to 30 gm. of urea in 1,000 c.c. of water by mouth at 6:00 A.M., less if blood urea nitrogen is above normal, giving enough to raise the blood urea nitrogen level to 28 mg./100 c.c.

2. Give no food but give 500 c.c. of water by mouth at 7:00, 8:00, 9:00, 10:00 and 11:00 A.M.

3. Have the patient void at 7:00 and 8:00 A.M. Discard these specimens.

4. Collect urine at 9:00, 10:00, 11:00 A.M. and 12:00 noon.

5. Collect blood at 9:30, 10:30, and 11:30 A.M.

6. Determine the ratio
$$\frac{\text{Urea N in 1-hour urine}}{\text{Blood urea N in 100 c.c. blood.}}$$

7. Take an average of the three ratios.

Concentration Test of Addis.†—Procedure.—1. The patient is instructed to abstain from fluids of all sorts after breakfast on one day until he arises from bed on the following day and is told to collect a twelve-hour specimen of night urine, voided directly into a wide-mouth bottle.

2. It must be made plain that the urine passed at the beginning of the twelve-hour period is to be discarded and only the urine saved which has been formed by the kidney during the twelve hours of the night.

3. Usual diet may be taken but water, tea, coffee, milk, soup, or any other fluids must be avoided.

In standardizing this test, Addis made 94 observations on 75 normal persons. The average specific gravity was 1.032; the standard deviation ± 0.00281 and the variability 8.8 per cent. Fig. 76 shows Addis' chart revealing the percentage frequency distribution of specific gravity.

*Addis, T., and Watanabe, C. K.: J. Biol. Chem. 28-29: 251-260, 1916, 1917, quoted from Belt, E.: loc. cit.

†Addis, T., and Shevky, M. C.: Arch. Int. Med. 30: 559, 1922.

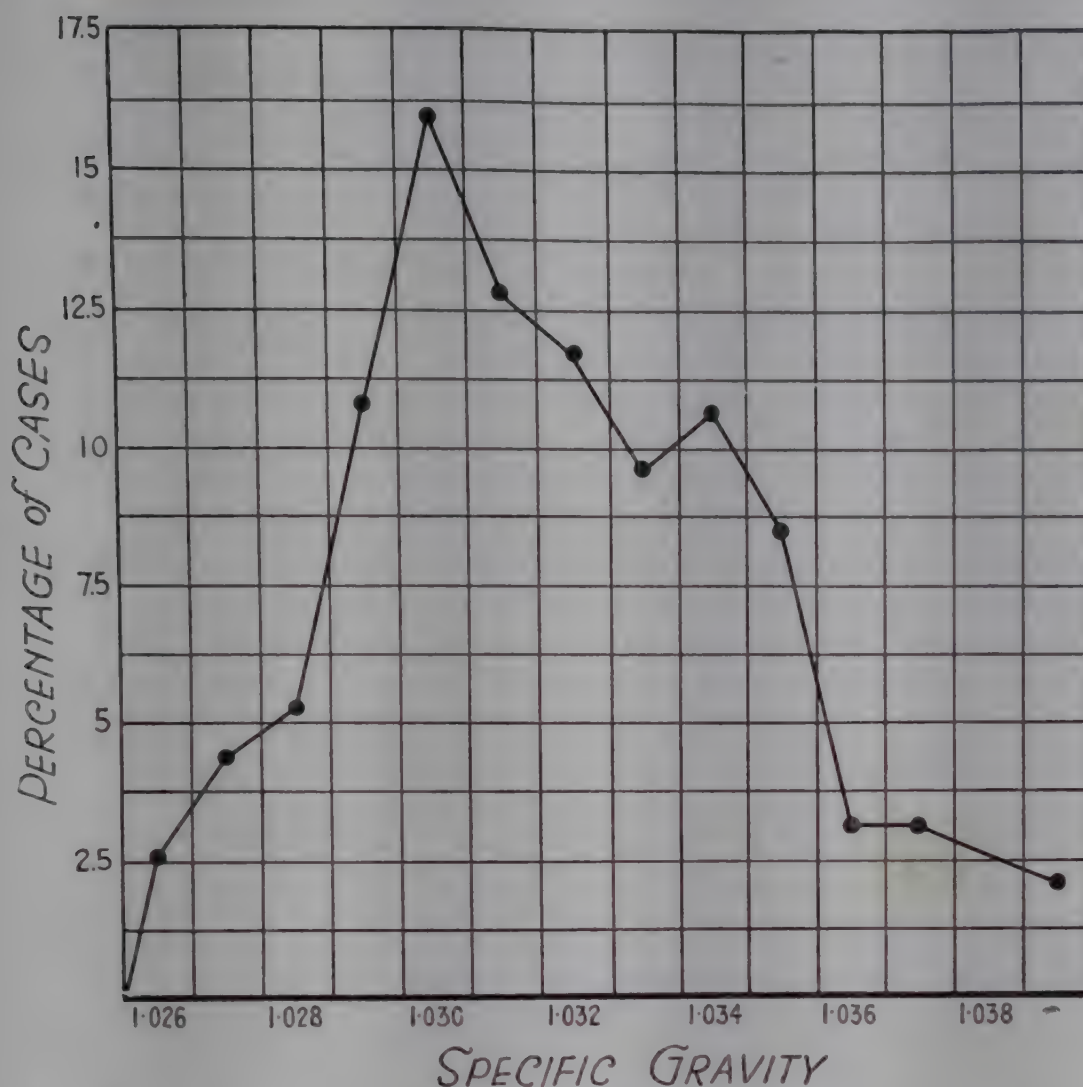


Fig. 76.—Percentage frequency distribution of specific gravity of the night urine in normal individuals after restriction of fluids. (Addis and Shevky.)

Addis Count of Urinary Sediment

The Addis count is a procedure to make the differentiation of the formed elements of the sediment of the urine more accurate. It was described by Thomas Addis in a communication entitled, "A Clinical Classification of Bright's Disease."¹

Technic.—

Secure the intelligent cooperation of the patient by written instructions given to him and spending some time with him to be sure he understands what is required. Let him take his usual breakfast next morning, including coffee, tea, or milk. Thereafter, he must abstain from all fluids during the day and night until the urine has been collected on the following morning. Fluids are defined as all substances that can be poured from one vessel into another. In other respects his usual diet is left unaltered, and he is specifically instructed not to take more fruit than is customary, even though he may become thirsty. The collection of urine may extend from any convenient hour in the evening until the patient rises from bed in the morning, say from 6 P.M. to 6 A.M. Be sure that the bladder is completely empty at the beginning and at the end of the collection. Note the time exactly. Unless there is a considerable volume of urine in the bladder, it often does not completely empty itself. The patient is, therefore, asked not to urinate during the afternoon of the day on which the collection is begun, and to void the total night urine when the collection is completed. The time at which the collection is begun and the time at which it is ended must be written by the patient on a label on the bottle into which the urine is voided.

¹Addis, Thomas: J. A. M. A. 85: 163, 1925.

Remember that the urine passed at the beginning must be discarded and not collected at all. The urine is passed into a wide necked bottle which has been thoroughly cleaned, washed with distilled water, dried in the inverted position, and finally rinsed with a clean solution of formaldehyde, which is allowed to drain out for a short time before the bottle is closed with a clean rubber stopper. In women, catheterization is essential for this procedure. The nurse must take pains to be sure that the bladder is really emptied, though even the possibility of an incomplete collection must be remembered. The patient returns the next morning, early, with the bottle of urine. It is almost always in a condition suitable for examination. If it is neutral or alkaline, there is a precipitate of phosphates. If the salt concentration is not too low, hyaline casts may still be found after the urine is cleared by the addition of concentrated acetic acid; but, as it is impossible to be certain that partial solution may not have occurred, cast counts are never made on such specimens, although the number of white blood cells and small round epithelial cells may be determined. If there is a precipitate of urates, the precipitate should be dissolved by placing the bottle in hot water.

Measure the volume of urine and return it to the bottle in which it was collected, reinsert the rubber stopper, and mix thoroughly by repeatedly inverting the bottle.

Immediately thereafter, 10 c.c. are transferred to a special graduated centrifuge tube with a diameter so narrow toward the tip that an accurate reading may be made.²

Centrifuge the tube for five minutes at 1,800 revolutions per minute.

In normal persons there is a deposit in the centrifuge tube of about 0.2 c.c. of what seems to be mucus. In the initial stage of the condition described as hemorrhagic Bright's disease, if the renal lesion is pronounced, there is a large amount of brown precipitate. In the active stage there are diminishing degrees of the brown color until in some cases it is imperceptible and the sediment is white. In the latent stage there is often no appreciable deviation from what is observed in normal urine. In the terminal stage there is no mucus; therefore, the cells and casts form a closely packed white deposit at the extreme tip, giving a characteristic appearance.

In degenerative Bright's disease, no brown color is ever to be seen and the amount of cloudy white deposit varies with the number of casts and epithelial cells. In arteriosclerotic Bright's disease there is simply the colorless mucus as obtained from the urine of normal persons. Decant the supernatant urine and remove part of the remainder with a pipette until it is reduced to a volume the magnitude of which will vary with the amount and nature of the deposit. In urines with appreciable concentration of protein, a precipitate appears at the surface of the urine as its meniscus approaches the narrowing tip of the tube. In such cases add a few drops of 1 per cent sodium chloride solution and again draw the fluid off until a convenient volume has been reached.

All the casts and cells from the 10 c.c. sample of the urine are concentrated in the volume that has been selected. Draw up and blow out the fluid with a capillary pipette, thus securing an even distribution of these elements. Add a drop into each side of a blood-counting chamber, and count the number of casts under the low power field over all the ruled areas. The area counted depends on the concentration of casts. In the urine of normal persons there are very few casts, and 10 drops are usually examined. When casts are numerous, the deposit is diluted to from 1 to 5 c.c., and only 2 drops are counted over a known area. The cells are counted under a high dry lens and usually over unit areas of 0.1 sq. mm. The differential cast count is made while the casts are being counted.

²Convenient and accurately made tubes are furnished by Arthur Thomas & Co., Philadelphia.

In the latent stage of hemorrhagic Bright's disease, when the number of casts is small, it is best to obtain a more concentrated mixture by centrifugalizing urine pipetted from the bottom of the collecting bottle after it has stood still for an hour or more.

Calculation.—

Since the chamber is $\frac{1}{10}$ mm. deep, the volume contained over the total ruled area is 0.9 c.mm., or 0.0009 c.c. If ten areas are examined, the volume of urine in which casts are counted is 0.009 c.c. Suppose that in this volume ninety casts are found and that the volume of urine or salt solution in which the sediment was mixed was 0.9 c.c. In that case $90 \times \frac{0.9}{0.009}$, which is 9,000, the number of casts in 0.9 c.c. But all the casts in 10 c.c. of urine were concentrated in that 0.9 c.c. volume, so 9,000 is the number of casts in 10 c.c. of urine. If the urine volume for twelve hours was 300 c.c., the number of casts in twelve hours is $9,000 \times \frac{300}{10}$, or 270,000. The same method of calculation is used in the cell counts. The general formula is as follows:

$$\frac{\text{No. counted} \times \frac{\text{Vol. in c.c. in which sediment was mixed}}{\text{Vol. in c.c. in which count was made}} \times \text{Vol. of urine in c.c. per 12 hours}}{10} = \text{No. in 12 hours' urine.}$$

The Addis Count in Children.—Boyle, Aldrich, Frank, and Borowsky¹ investigated the usefulness of the Addis count in estimating recovery of children from postinfectious nephritis. The Addis count, described by Thomas Addis² was based upon the idea that the type of renal pathology might be predicted from study of the sedimentation. In 1926,³ he published what he considered to be normal figures for the various constituents, particularly red blood cells, leukocytes, epithelial cells, and casts. Goldring⁴ made studies of the sediment of clinically well adults and of adults with nephritis, rheumatic heart disease, subacute bacterial endocarditis, and pneumonia. His averages for normal adults were higher than those of Addis. Naeraa⁵ reported figures for normal adults higher than those of Addis and lower than those of Goldring. He claimed that the number of red blood cells increases with the age of the individual. The present writers studied 250 children who had had acute post-infectious hemorrhagic nephritis and had made a clinical recovery. Twenty-five, or 10 per cent, were selected for this study. The methods used for collecting specimens and making the counts were those described by Addis, as modified by Lyttle for children. Fluids were rigidly restricted during the day preceding the collection of urine; then all the urine passed between 7 P.M. and 7 A.M. was collected in one container. A definite quantity of the urine was centrifugated at a given speed for a given length of time and a quantitative estimation of the formed elements was made in a counting chamber. From this figure the total number of cells and casts in the twelve-hour specimen was calculated.

¹Boyle, H. H., Aldrich, C. A., Frank, Albert, and Borowsky, Sydney: *J. A. M. A.* 108: 1496, 1937.

²Addis, Thomas: *J. A. M. A.* 85: 163, 1925.

³Addis, Thomas: *J. Clin. Invest.* 2: 409, 1926.

⁴Goldring, Wm.: *Am. J. M. Sc.* 182: 105, 1931; *J. Clin. Invest.* 10: 355, 1931.

⁵Naeraa, Asger: *Hospitaltid.* 77: 1445, 1934.

The results on these postinfectious hemorrhagic nephritis cases showed normal counts, proving that the patients did not have subacute or latent nephritis. Clinical recovery in this instance implied absence of all known symptoms and signs of nephritis, as well as completely negative urinalyses as routinely conducted.

The results of the examination of the urinary sediments of the patients who were investigated by Addis fall into classifications in consonance with the classification of Bright's disease used by him. He calls the first division hemorrhagic Bright's disease, the second degenerative Bright's disease, and the third arteriosclerotic Bright's disease.

Hemorrhagic Bright's Disease.—This is the glomerulonephritis of the pathologists. In this condition the urine contains so many red blood cells that it has a mahogany brown appearance. The precipitate consists of lysed and distorted red blood cells, with a considerable admixture of pus cells and epithelial cells. Casts found in this cellular débris vary from lemon yellow to dark brown in color. Red blood cells are found. Blood casts are found which are pathognomonic of hemorrhagic Bright's disease. The symptomatology of this disease is quite variable, but there is usually a moderate increase in diastolic pressure and often a slight, generalized edema. Since these cases are usually due to streptococcic infection, with the subsidence of the infection, as for instance in scarlet fever, there is a rapid decline in the activity of the renal lesion and ultimately healing, with a defect that is compensated for by hypertrophy. The latent stage is due to focal streptococcal infection. With the exception of the indications to be obtained from the sediment, it is entirely latent, and even the urine may be passed as normal unless it is concentrated.

The active stage follows the initial stage, or it may be the result of an exacerbation of the continuing streptococcal infection in a patient with latent hemorrhagic Bright's disease. It is accompanied by an increase in the number of casts and in the rate of protein excretion and by the appearance of fatty casts and fat droplets on all the hyaline casts. In untreated cases there is general anasarca. If there is no increase in blood pressure, it is only by the large number of red blood cells and the finding of an occasional blood cast that the condition can be distinguished from what Volhard and Fahr¹ called "genuine nephrosis." With the disappearance of the infection the lesion may become latent and heal; with its continuance it will progress, either directly or after an interval of latency, to the terminal stage.

As the terminal stage approaches, there is a gradual change in the nature of the urinary sediment. As one after another of the inflamed glomeruli become completely disabled and fibrosed so that urine no longer flows down their tubules, fewer red blood cells and pus cells come from the shrunken kidney, and blood casts are hard to find. When the amount of secreting tissue is decreased to less than one-third of its original amount, the urea concentration of the blood begins to rise; and, as the kidney grows smaller and smaller, the blood urea concentration rises higher and higher until under its influence the small remnant of renal tissue is secreting a urine that is constantly dilute. All the casts are renal failure casts.

¹Volhard, F., and Fahr, T.: *Die Brightsche Nierenkrankheit*, Berlin, 1914, Julius Springer.

Degenerative Bright's Disease.—This second classification has been called degenerative Bright's disease because the most prominent feature of the sediment is the large number of epithelial cells in various stages of granular or fatty degeneration. It is impossible to determine whether they were originally epithelial or white blood cells. Addis counts them together.

Degenerative Bright's disease is not a unity but only a convenient term under which to group all those many pathologic conditions which may give rise to degenerative changes in the kidneys. They may be summarized under six divisions.

1. Certain cases of unknown etiology are called cryptic degenerative Bright's disease. These cases are usually seen only when edema has developed. Nothing is known of the initial stage.

2. There are patients with degenerative Bright's disease in whom the chemical constitution of the causative agent is known, such as mercury, chromium, and uranium salts, as in jaundice and in some cases of malaria.

3. The unknown toxin derived from the fetus in the latter half of pregnancy produces a degenerative Bright's disease accompanied by hypertension.

4. Toxemia that may accompany any generalized infection is likely to produce a slight cloudy swelling of the kidney. This may take the form of desquamation of renal cells with many epithelial and granular casts and much protein.

5. Focal infections are not often associated with any but minor grades of renal degeneration, but some patients develop proteinuria and edema. This type has been described in children by Clausen.¹

6. Mixed infections which are found in long-standing osteomyelitis with sinus formation tend to produce the waxy kidney.

Arteriosclerotic Bright's Disease.—This is the final classification of Addis. It occurs more commonly than any other form of Bright's disease. So far as the kidney is concerned, it is of negative character; for, when the diagnosis has been made, it is no longer necessary to consider the renal lesion as a factor in the management of the patient. These are the patients with hypertension who are often told that they are suffering from "chronic interstitial nephritis" and who consequently live in fear of death of a uremia that never comes. With examination of the urinary sediment by Addis' method, it is possible to construct a clear picture of the genesis, the rise and fall, and, if fibrosis advances, the terminal diminution in the activity of the renal lesion; the classification that grows out of such observations is an advance toward a purely etiological classification, the classification that must always remain the one toward which we strive.

Xanthoproteic Reaction

Volhard termed the xanthoproteic test a criterion for judging the danger to life of the kidney lesion. It was assumed by Beecher that the symptoms of uremia parallel the accumulation of products of intestinal putrefaction, such as phenol, phenol derivatives, paracresol, and oxy acids in the blood. These undetoxified products of intestinal decomposition accumulate in the blood and

¹Clausen, S. W.: *Am. J. Dis. Child.* 29: 581, 1925.

tissues because the detoxifying processes are no longer carried out completely in the wall of the intestine and the liver. The chromogen materials are retained in the skin and these give the yellow color to the skin of uremics.

Technic.¹—

To 2 to 3 c.c. of the solution to be tested, add concentrated nitric acid.

Upon the addition of nitric acid a white precipitate forms which disappears; the solution turns yellow upon heating.

Cool and add ammonium, potassium, or sodium hydroxide. The color of the yellow solution deepens to orange.

This chemical reaction is due to the phenol group with which nitric acid forms nitro modifications. Hawk criticized the test as not satisfactory for use in urinary examinations because of the indeterminate color of the end point, therefore making it qualitative only.

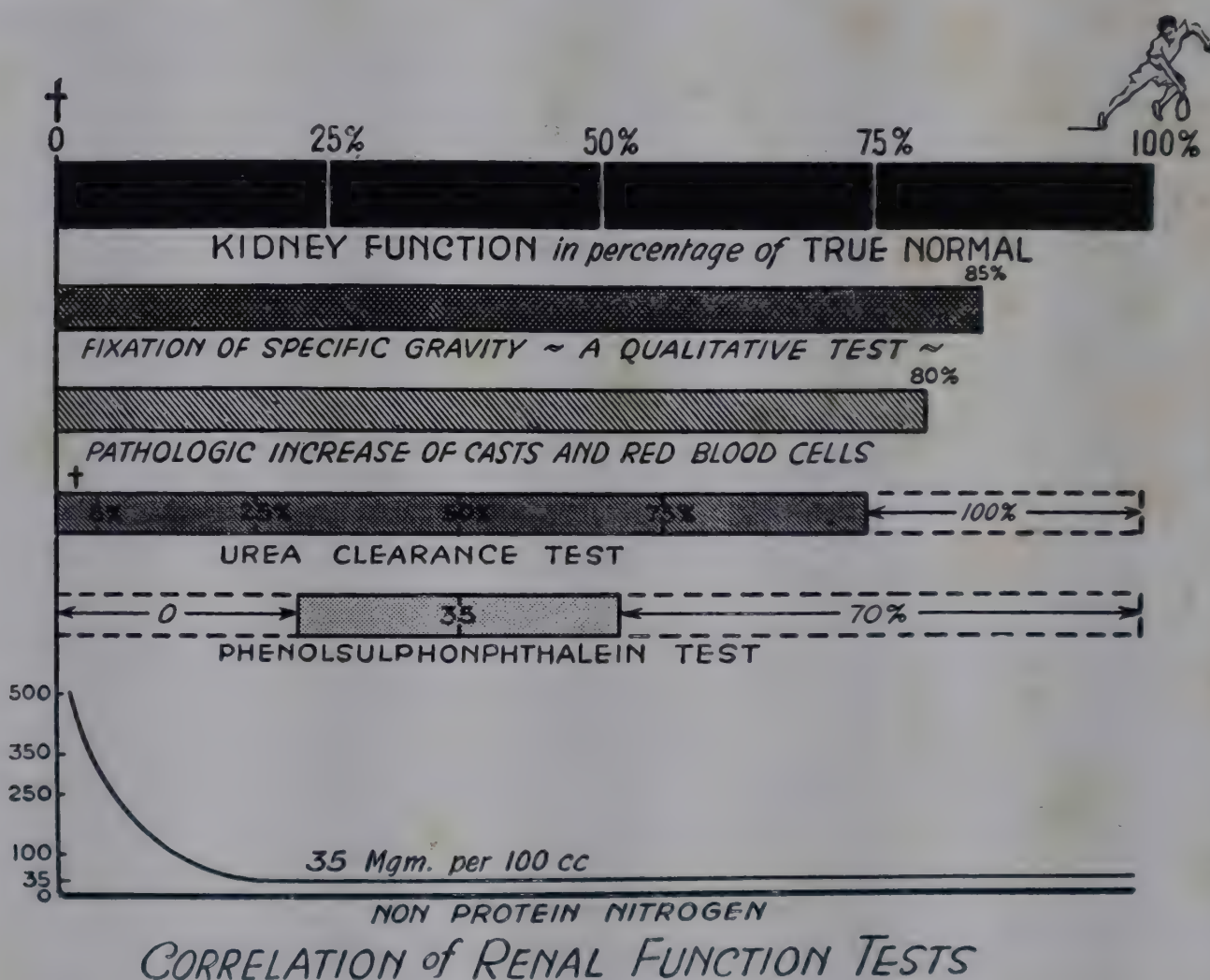


Fig. 77.—The solid bar at the top represents the true kidney function. This is never completely measured by any test. The tests in current use are represented below by a series of shaded bars. The length of each indicates its range in comparison with the others and with the true normal. (Courtesy of Belt: Cabot's Modern Urology, Lea and Febiger.)

Correlation of Renal Function Tests¹

It is not often possible to correlate the various tests which are supposed to express kidney function in per cent of normal. As Belt² has summarized in his excellent review of this subject, readings taken at the same time in the same individual may be quite low in one type of test and definitely high in another. On the other hand, a very close correlation will be found to exist

¹See section on urea clearance, etc., pages 162, 261, and 279.

²Belt, E.: Modern Tests in Renal Function, Cabot's Modern Urology, Vol. II, Philadelphia, 1936, Lea & Febiger.

between different readings of the same test done on successive days. This discrepancy arises through the fact that each particular test must be an expression of a slightly different phase of kidney function. In order to give a graphic expression of these facts, Belt has compiled a chart (Fig. 77) which shows that the earliest sign of renal dysfunction is a fixation of specific gravity. This test is extremely sensitive in revealing slight renal damage, but it does not distinguish between a moderately advanced and a terminal stage of the disease. He states that an abnormal number of casts and red cells is the next most sensitive indication of disorder. This has a slightly quantitative significance when differential counts are compared from time to time. He calls attention to the fact that the urea clearance test has the widest range of all the tests attempting to express function in per cent of normal. It is not sensitive to changes less advanced than are represented by a loss of 20 per cent of the kidneys' functional capacity but does permit readings on the low side of the scale all the way down to 5 per cent.

Furthermore, the phenolsulphonephthalein test is less sensitive to early changes than the urea clearance test. Zero readings are recorded by it when a wide margin of kidney function still exists.

The nonprotein nitrogen begins to be elevated as the phenolsulphonephthalein test approaches zero and is always high when the urea clearance test is 20 per cent or below. In the absence of general metabolic factors which might influence the degree of elevation of the nonprotein nitrogen, its level above normal marks the diminution of secreting tissue toward zero.

INTERPRETATION OF URINARY FINDINGS IN VARIOUS CONDITIONS

Chyluria

There are two kinds of chyluria: first, the tropical form due to invasion of the blood and urinary tract by the *Wuchereria bancrofti*; second, a variety that is not parasitic but of unknown etiology. The disease is the result of lymphatic obstruction. The urine is of an opaque white color, closely resembling milk; under the microscope the turbidity seems to be caused by numerous minute granules, the fat being in molecular form. The only difference between lymph and chyle is that the latter contains a much larger percentage of fat.

The generally accepted theory of the cause of chyluria holds that there is a fistulous communication between the lymphatic and urinary tracts, and presupposes a lymphatic obstruction somewhere between the small intestines and the thoracic duct. Chyluria occurs in the presence of thoracic duct obstruction only when the collateral lymphatic circulation is inadequate.

The lymphatic vessels of the kidney form three plexus: one in the substance of the kidney, a second beneath its fibrous capsule, and a third in the perinephric fat. The second and third communicate freely with each other. The vessels from the plexus in the kidney substance converge to form four to seven trunks which issue at the hilum. Here they are joined by vessels

from the plexus under the capsule, and, following the course of the renal vein, end in the lateral aortic glands. The perinephric plexus is drained directly into the upper lateral glands.

The route from the intestinal to the renal lymphatics has been the subject of much speculation. According to Magnus-Levy, the chyle must first go from the mesenteric lymph channels through the mesenteric lymph glands to the thoracic duct. Then, because of obstruction, there is a retrograde flow to the upper lumbar lymph glands which drain the renal lymphatics. To obtain such a retrograde flow, an insufficiency of the valvular system of the lymphatic channels must be assumed. Many explanations have been offered for chyluria, but the theory of lymphatic block and direct urinary tract communication is probably the most sufficient for the present. Hampton¹ succinctly sums up the matter as follows: "I believe that the kidney lymphatics represent the point of lowest resistance in the lymphatic system and when the thoracic duct or large trunks leading from the kidneys are blocked, they rupture just as an esophageal varix does under portal obstruction, or as the cerebral arteries do in hypertension cases. It seems evident that the deep lymphatics of the kidneys are involved and they rupture into the tubules or directly into the calices."

Symptoms of Chyluria.—The symptoms of chyluria are mainly the milky appearance of the urine, which suddenly follows the partaking of a meal rich in fats. It may develop after a strain or it may appear without any demonstrable cause. It has been noted during childbirth with subsequent cessation after delivery. It may be continuous or intermittent. In some cases, it is present at all times; some have observed it in only the night urines, clearing as the patient became active during the day; still others noted chyle in the day urine only; again, it was found only when the patient had been exercising vigorously; it may be influenced by posture.

Lipuria and marked *pyuria* may be confused with chyluria; in the former the fat is present in the droplet form although occasionally emulsified to the extent of a milky color. Microscopic examination will differentiate the pyurias. The fat in the urine of chyluria is in molecular form and, therefore, presents no morphology. In such cases the presence or absence of fibrinogen, cholesterol, or lecithin will be of significance.

In chyluria the urine contains coagula, due to the large amount of fibrin. These clots may form in the bladder and be abundant enough to give rise to symptoms. The specific gravity is low, reaction is acid, and there is a large amount of albumin. Microscopically, the clots show fibrin with a number of red cells embedded with small plates of hematin. There are many red cells and many small fat globules. Pus corpuscles and epithelia from the ureters and bladder are found. In the tropical variety, one may see the embryonal forms of the filaria.

Two glasses of equal parts milk and cream should cause chyle to appear in a catheterized specimen of urine removed at the end of one hour. A second specimen should be taken at the end of four hours. If the test is negative, it

¹Hampton, H. H.: Bull. Johns Hopkins Hosp. 31: 20-24, 1920.

should be repeated with the patient in the recumbent position. Since chyluria is intermittent, all the tests may be negative, but if injected perirenal lymphatics are present and the tests are repeated at intervals, chyluria will undoubtedly be found. Hampton kept his patient on Johns Hopkins Hospital diet, adding an extra amount of butter and giving two glasses of milk before each test. He catheterized the kidneys every hour for four hours. Chyle was present in large amounts in the first-hour specimen and had practically disappeared in the last collection.

Renal Tuberculosis

In early cases of renal tuberculosis the urine may be practically normal in appearance, but it is frequently cloudy from pus. The volume may not be affected, but is likely to be increased, the reaction is acid, and there are traces of albumin and a few renal cells. In advanced cases the urine is usually pale, cloudy, and alkaline, has an offensive odor, and is irritating to the bladder. In such cases albumin and pus are always present, though frequently not abundant. The pus is generally intimately mixed with the urine and does not settle as quickly as does the pus of cystitis. Casts are seldom abundant and are obscured by the pus. Tubercle bacilli are nearly always present even when the pus is extremely slight and their detection is essential for the diagnosis. In some cases they may be found by appropriate staining of the sediment after prolonged centrifugalization. In other cases they cannot be found, and it is necessary to inoculate guinea pigs to make the diagnosis.

Hirschberg¹ has discussed the demonstration of tubercle bacilli in the urine. The guinea pig inoculation method has been criticized because many guinea pigs contract tuberculosis spontaneously or have internal lesions which resemble those caused by the tubercle bacillus. Howell and Schultz² report an epizootic among guinea pigs due to a paratyphoid B bacillus which caused lesions closely resembling tubercles in the spleen and liver. Magath and Feldman³ conclude that with reasonable care spontaneous tuberculous infection of guinea pigs can be avoided. Cummings⁴ reported the incidence of spontaneous tuberculous infection in laboratory animals in Saranac, a tuberculosis center. Hirschberg attempted to make comparisons between the direct smear, the guinea pig inoculation and culture methods for the demonstration of tubercle bacilli in the routine specimens received in the laboratory of a large hospital, reaching the conclusion that for the demonstration of tubercle bacilli in the various types of specimens, the culture method gives a greater percentage of positives more quickly than either of the other two methods. Of the various mediums used for the isolation of the tubercle bacilli, Corper's, Hohn's and Miraglia's were found to be suitable for routine diagnostic cultures. The Hohn medium is made by the addition of a small amount of methyl green to sterile liquid egg white. Corper's medium is made according to his

¹Hirschberg, N.: *J. Lab. & Clin. Med.* 19: 429, 1934.

²Howell, K. M., and Schultz, O. T.: *J. Infect. Dis.* 30: 516, 1922.

³Magath, T., and Feldman, W. H.: *Am. J. Clin. Path.* 2: 199, 1932.

⁴Cummings, D. E.: *Am. Rev. Tuberc.* 26: 388, 1932.

standard method. Special culture media for culture of tubercle bacilli are described in Chapter X. Petrik's medium is excellent and is highly recommended. More recent media are Tarshis and Whalen.

Corper's Standard Method of Cultivation of Tubercle Bacilli.—Prepare Corper's glycerol water crystal violet paper medium by cutting large clean potatoes into cylinders which are soaked from one to two hours in a 1 per cent sodium carbonate solution, which contains crystal violet in concentration of 1 to 75,000. This dye must be added just before use, since the mixture tends to decolorize. These potato cylinders are wiped clean and placed in sterile tubes containing 1.5 c.c. of 6 per cent glycerol water and sterilized at 120° C. 15 pounds pressure for thirty minutes.

(See Chapter X for a further description of tubercle bacilli in urine.)

Malignant Tumors of the Kidneys

In malignant tumors of the kidneys, we often find hematuria, intermittent or constant, as a urinary sign. In about three-fourths of the cases, it is the first definite symptom.

Renal Calculus

In stone of the kidney, the urine is somewhat concentrated, with high color and strongly acid reaction. Small amounts of albumin and a few casts may be present as a result of kidney irritation. The finding of blood cells is a very important suggestive fact in this disease, especially during the day and after severe exercise. The presence of a stone usually produces pyelitis, and variable amounts of pus then appear, the urine remaining acid in reaction. Diagnosis is confirmed by catheterization, x-ray, etc.

Pyelitis

In pyelitis the urine is slightly acid, and contains a small or moderate amount of pus, together with many spindle and caudate epithelial cells. These findings may be intermittent, owing to occasional blocking of the ureter on the diseased side. Pus casts and also other forms may appear when the process extends up into the kidney tubules. Albumin is always present. Even when pus is scanty, albumin is rarely under 0.15 per cent. In all these cases, of course, bacteriologic studies must be made. *Proteus vulgaris* is found in about 50 per cent of the cases. In other cases, we find staphylococci.

Cystitis

In acute and in many chronic cases of moderate severity, the urine is acid and contains a variable amount of pus, with many epithelial cells from the bladder, which are large, round, and pyriform cells. Albumin is present in small amount—less than 0.15 per cent. *Escherichia coli* is most frequently found in these cases. In old chronic cases, especially when due to prostatic obstruction, the urine is alkaline. The sediment contains amorphous phosphates and crystals of triple phosphate and ammonium biurate. Many organisms are present.

Stone in the Bladder, Tumors, and Tuberculosis

These conditions will produce chronic cystitis, with its characteristic urine. Blood is more frequently present and more abundant than in ordinary cystitis. With neoplasms, especially, considerable hemorrhage is apt to be found and sometimes particles of the tumor are passed with the urine. In cases of tuberculosis, it is comparatively easy to find tubercle bacilli.

Diabetes Insipidus

Diabetes insipidus is characterized by an excretion of increased quantity of pale watery urine. The specific gravity is very low, around 1.001. The urine shows no sugar. There is, however, a marked increase in urea in many cases of diabetes insipidus.

Diabetes Mellitus

Tests for diabetes mellitus are given in the chapter on Blood Chemistry, pages 237 ff. The quantity of urine is increased up to as high as four liters in twenty-four hours. The urine is pale. The specific gravity is high, around 1.030 to 1.040. The urine shows the presence of dextrose to a greater or less extent. Blood sugar is above the normal, 0.12 per cent. The quantity of dextrose in the urine varies from 2 to as much as 8 per cent. In advanced cases acetone is present; in very bad cases diacetic acid and oxybutyric acid appear.

Renal Diabetes

Renal diabetes is discussed under Blood Chemistry, page 242. In this disease there is no increase in blood sugar. There is a condition of lowering of the threshold point; that is to say, instead of no sugar appearing in the urine until the blood sugar reaches 0.17 per cent or more, sugar appears in the urine with perfectly normal blood sugar content. It seems to be a disease of the filtration process of the kidney, or represents a disturbance of the permeability of the kidney for sugar.

Nephrosis and Nephritis

The meaning of the term *nephritis* in the light of present knowledge of pathology of the kidney has been changed to mean only those conditions which are *inflammatory* in nature. The primarily *degenerative* forms of kidney disease are called *nephroses*. The arteriosclerotic kidney, which is a result of changes in the kidney due to arteriosclerosis of the renal arteries, is not classified as nephrosis. Amyloid disease, which starts as a degenerative change in the vessels, is a nephrosis.

Nephroses are those diseases characterized anatomically by primarily degenerative lesions of the renal parenchyma. According to Fishberg,¹ nephroses may be divided into four groups as follows:

1. Larval nephroses, in which the renal lesions are of comparatively slight degree and are manifested clinically by albuminuria and the presence of some formed elements in the urine in the absence of impairment of renal function.

¹Fishberg, Arthur M.: Hypertension and Nephritis, page 299. Philadelphia, 1934, Lea & Febiger.

2. Necrotizing nephroses, in which there is necrosis of the tubular epithelium. In these cases renal insufficiency is common.

3. Chronic nephroses, in which there is chronic degenerative process in the renal epithelia, with edema as the most striking clinical feature.

4. Amyloid nephrosis.

Among the larval nephroses may be catalogued febrile albuminuria, diabetic nephrosis, nephrosis due to long-standing jaundice, pernicious anemia, and Graves' disease.

Of the necrotizing nephroses we are concerned with that type seen in acute mercury poisoning.

Chronic nephrosis is characterized clinically by edema and severe albuminuria with no arterial hypertension. Anatomically in these cases are found degenerative lesions of the renal parenchyma. Chronic nephrosis is the present term for what was formerly called parenchymatous nephritis, the large white kidney of the pathologic anatomist.

The etiology of chronic nephrosis is as follows: It is a disease of childhood and young adult life, decreasing in frequency with advancing years. Infection is supposed to play an important rôle in this condition. Of all the infectious agents, the *Treponema pallidum* is the most clearly defined. Diphtheria, as well as pneumococcus infections, has been known to have been complicated by chronic nephrosis.

In chronic nephrosis we find edema, proteinuria, diminution in the colloid osmotic pressure, lipemia, no impairment of renal function, arterial hypertension, and no primary inflammatory lesions of the kidney.

Albuminuria is the most common feature of chronic nephrosis. Slight and temporary glycosuria is sometimes found. In the sediment we find doubly refractile lipoids, first described by Munk. In order to determine the presence of these bodies, we must use a polarizing device which can be attached to any microscope. In the blood we find a decrease in the total protein content of the plasma, increase in the fibrinogen content of the plasma, decrease in the colloid osmotic pressure of the plasma, increase in the velocity of sedimentation of the red blood cells, increase in the concentration of fats and lipoids in the plasma.

Amyloidosis.—In amyloid nephrosis the amount of protein in the urine is very great; as much as 30 grams of protein may be lost in the urine daily. The blood picture in these cases shows profound anemia. There is sometimes a hypercholesterinemia. Edema is a common clinical symptom.

Congo Red Test for Amyloidosis.—

In cases of amyloidosis, when this colloidal dye, Congo red, is injected into the blood stream, it is removed from the plasma by the amyloid because amyloid substances have a special attraction for Congo red. *When amyloid is not present*, most of the dye remains in the blood stream and is found there one hour later.

Bennhold¹ has described a test for amyloid. If a colloidal suspension of the dye Congo red is injected intravenously, less than 30 per cent of it dis-

¹Bennhold, H.: Deutsches Arch. f. klin. Med. 142: 32, 1923; Klin. Wchnschr. 3: 1711, 1924.

appears from the blood stream of normal persons within an hour. In the presence of amyloid disease from 40 to 100 per cent leaves the blood stream within an hour.

A simplification of this Bennhold test has been worked out by Cohen.² It is simpler than the Bennhold test because it requires only one vein puncture instead of three, as required by the Bennhold test.

Technic of Congo Red Test.—

Inject 2 c.c. of a 1% Congo red solution intravenously, and after exactly fifteen minutes withdraw a few drops of blood from a finger into a suitable tube with capillary mouth. After the blood has clotted, centrifuge at slow speed. Remove one or two drops of clear serum with a capillary pipette and place in a small white porcelain dish. Add one drop of undiluted hydrochloric acid with another capillary pipette. If the serum contains Congo red, a blue, quickly fading color appears at the moment of mixing.

Congo Red Quantitative Test.—

Inject 1.2% solution of Congo red, 0.25 c.c. per kilogram of body weight.

After 5 minutes and again after 1 hour (65 minutes altogether), withdraw blood and compare the plasma in a colorimeter.

$$\frac{\text{Reading at 65 minutes}}{\text{Reading at 5 minutes}} \times 100 = \% \text{ of the dye remaining in the plasma. Normally 70 to 90\% remains after 65 minutes.}$$

This modification is based upon the rapid initial absorption of Congo red in the presence of amyloid, the slow initial absorption of Congo red in persons without amyloidosis, and the easy demonstration of Congo red in a dilution as low as 0.00025%. It is estimated that when 2 c.c. of 1% solution of Congo red are injected in a normal person weighing 150 pounds, the total plasma, or 2,700 c.c., would contain about 0.00074% of Congo red, or three times the demonstrable concentration.

Expressed in other terms, the Cohen modification simply shows whether or not Congo red has been retained. In the original Bennhold test, a colorimetric comparison is made of the content of Congo red in the serum four minutes after it is injected and at the end of sixty minutes.

Gerber and Fryczynski³ showed that a serious source of error in this test is that of hemolysis, due to too rapid withdrawal of the blood, the forcible ejection of blood from the syringe, the use of a wet syringe or needle, inadequate mixing of the blood with the anticoagulant and the improper withdrawal leading to the formation of small clots in the syringe. These errors may all be avoided; in addition, these workers suggest the use of heparin as an anticoagulant rather than any other anticoagulant. They suggest the use of the sodium salt of heparin (Liquaemin*). One-tenth of a cubic centimeter suffices to prevent the coagulation of ten to twelve cubic centimeters of blood. Blood after withdrawal is immediately rotated in a clean bottle containing the Liquaemin. It can be allowed to stand, without any fear of hemolysis. Centrifuge the blood for 10 to 15 minutes at 1,500 r.p.m., take off the plasma, and match in a micro-colorimeter. Absence of hemolysis facilitates colorimetric readings.

Selikoff⁴ has made a thorough study of the Congo red test in generalized amyloidosis and confirms the original work; namely, that this test is a valuable

*Liquaemin furnished by Roche-Organon, Inc., Nutley, N. J.

²Cohen, A. B.: J. Lab. & Clin. Med. 27: 934, 1942.

³Gerber, I. E., and Fryczynski, M.: Am. J. Clin. Path. 12: 312, 1942.

⁴Selikoff, I. J.: Am. J. M. Sc. 213: 719, 727, June, 1947.

aid in the diagnosis of amyloidosis. Where only small deposits of amyloid substance are present, there will be failure to absorb appreciable amounts of Congo red. This will lead to "false negative" results. Also patients with small amounts of amyloid may show 0 to 89 per cent absorption. This test does not differentiate between these cases and those without amyloidosis and in this range has no diagnostic value. It has also been shown that cases occur which show 90 to 100 per cent absorption and yet have no amyloid. These are "false positive" tests. This author concluded that in such cases there should be complete or nearly complete absorption in *two* consecutive Congo red tests in order to consider a patient amyloid.

Nephritis.—(See pages 174 ff. for further discussion of this condition.) The type of nephritis which is most common is the acute glomerulonephritis, due to infection, such as scarlet fever, tonsillitis, subacute bacterial endocarditis, pneumonia, purpura, rheumatic fever, influenza, tuberculosis, and trench mouth.

The urine is diminished, blood is present, and albuminuria is present in all cases, usually from 0.2 to 0.4 per cent. In the sediment, in addition to red cells and urates, we find casts—hyaline and blood casts, sometimes fatty and rarely waxy casts—leukocytes in great numbers, and epithelial cells. Specific gravity of the urine is high, due to prerenal deviation of water into the tissues to form edema. There is usually impairment of renal function with retention of the nonprotein nitrogenous constituents. Nonprotein nitrogen of the blood is usually not high, but may be as high as 180 mg. per 100 c.c. In some cases concentration of chloride in the blood is high, in others, low. Total protein content of the plasma is within normal limits.

Chronic Glomerulonephritis.—This is a condition of an unresolved acute glomerulonephritis. It usually follows cases due to angina or exposure to cold; sometimes it is seen in subacute bacterial endocarditis if the patient lives long enough. There are three types of chronic glomerulonephritis, the nephrotic type with edema, the hypertensive type, and the recurrent type. The amount of urine is decreased, and the specific gravity is low. The nonprotein nitrogenous constituents in the blood show no changes so long as renal function is unimpaired. With the beginning of renal decompensation retention occurs. Albuminuria is constantly present. Casts are found in the urine of all varieties, hyaline, granular, epithelium, and fatty. Blood is frequently present.

Focal Nephritis.—According to Fishberg, the term focal nephritis is used to indicate a nephritis which is a manifestation of infection, occurring at the height of the infection. Fishberg emphasizes, as Volhard¹ has already pointed out, an important difference between focal nephritis and acute glomerulonephritis, "for the latter most often, though not invariably, occurs after the primary infection has started to subside, as is seen in glomerulonephritis complicating scarlet fever and often tonsillitis. The close linkage of focal nephritis to the height of the infection is well illustrated by the old observation of Kannenberg² that in focal nephritis complicating relapsing fever the renal process waxes and wanes with the periods of the disease." It is probable that the lesion in focal nephritis is due to the direct action of microorganisms on the renal structures.

¹Volhard: In Mohr and Staechelin's *Handb. d. inn. Med.*, Berlin 8: 152, 1918.

²Kannenberg: *Ztschr. f. klin. Med.* 1: 506, 1880.

The types of infection in which this condition occurs are as follows: streptococcal infections, pneumonia, typhoid fever, and rarely in scarlet fever, measles, cerebrospinal fever, influenza, rheumatic fever, and many others.

In these cases hematuria is the leading symptom, occurring at the height of the infectious process. Together with blood in the urine, we find albumin, moderate in quantity, and varying numbers of hyaline, granular, and blood casts. Leukocytes are abundant, and epithelial cells may be present. In cases complicating tonsillitis, we are likely to find bacteria in the stained sediment. Renal function is usually not impaired to any great degree, although the volume of urine is diminished.

Nephropathies Due to Vascular Disease.—In the majority of elderly persons there is some sclerosis of the renal arteries which extends into the prearterioles. This form of renal disease is called the arteriosclerotic kidney, and it may be found in persons who have had no signs of renal disease or hypertension.

Arteriolosclerotic or Hypertensive Kidney.—In this condition there is disease of the small arteries and afferent glomerular arterioles, with persistent elevation of the blood pressure. The patient sooner or later develops signs of involvement of the heart, brain, or kidneys. In these cases death occurs from congestive heart failure, coronary disease, apoplexy, or renal insufficiency. Autopsies on many cases of primary hypertension have shown normal-sized kidneys with finely granular surfaces. The so-called “primary contracted kidney” is a hypertensive condition with a high degree of atrophy. One must distinguish chronic glomerulonephritis from primary hypertension with uremia since the two diseases are different in etiology.

Urinary 17-Ketosteroids

See Chapter IX, Special Tests, for a discussion of urinary 17-ketosteroids.

CHAPTER III

BLOOD CHEMISTRY

Blood chemical procedures are very important in helping physicians in diagnosis, prognosis, and treatment. An understanding of the normal composition of the blood is vital in order properly to interpret abnormal findings. Table 9 gives the composition of normal blood in milligrams per 100 c.c. and the pathologic conditions in which increases may be encountered. A study of this chart will be very helpful in properly utilizing reports on blood chemical findings. Physicians should be instructed that these chemical analyses are all based upon a period of fasting and the specimens should be taken in the morning, preferably before breakfast. The specimens should be delivered to the laboratory as promptly as possible, especially in the case of blood sugar and prothrombin time determinations since changes occur very quickly and interfere with the accuracy of results and interpretations. Some examinations require whole blood while others are conducted with plasma or serum. If whole blood is collected, an anticoagulant should be used. (See page 191 for the various anticoagulants.) If serum is to be used, the blood is collected in a clean, dry, sterile test tube and allowed to clot.

The four protein constituents in blood are serum albumin, euglobulin, pseudoglobulin, and fibrinogen. Nucleoprotein is also present. Total protein in the plasma remains relatively constant in normal individuals. Values for human plasma of total protein and its various factors are as follows, estimated in grams per 100 c.c.:

Total protein	6 to 8 gm. per 100 c.c.
Fibrinogen (plasma)	0.2 to 0.4 gm. per 100 c.c.
Globulin (serum)	1.5 to 3.2 gm. per 100 c.c.
Albumin (serum)	3.5 to 5.6 gm. per 100 c.c.
Ratio of the three factors (approximate)	1:4:18
Albumin-globulin ratio (A/G)	1.5:1 to 3:1

Definite changes occur in the total plasma protein content from time to time as a result of reduction in the water. Fibrinogen is increased under parathyroid overdosage and when the tissues are markedly injured. Certain derangements of the liver are accompanied by marked reduction of this constituent. It is also reduced in phosphorus poisoning and acute yellow atrophy. The total protein is reduced in nephritis and often in cases of malnutrition. The reduction of the total index is due to the reduction in the albumin.

Attention must be called to the difference between blood serum and blood plasma. The protein element found in plasma and not present in serum is fibrinogen, which is transformed into fibrin during coagulation and removed.

Normal blood contains 20 per cent of solids and 3 per cent of total nitrogen, whereas chlorides are present in the blood to the extent of 0.45 to 0.5 per cent. Chlorides are decreased in diabetes because of accompanying diuresis and increased in some forms of nephritis due to retention.

TABLE 9.—COMPOSITION OF HUMAN BLOOD*

CONSTITUENT	NORMAL RANGE MG. PER 100 C.C. ¹	PATHOLOGIC CONDITIONS IN WHICH IN- CREASES (UNLESS OTHERWISE NOTED) MAY BE ENCOUNTERED
Total solids, per cent	19—23	Anhydremia. Low in hydremic plethora and anemia.
Total proteins (serum) per cent	6 to 8 Gm.	See above. Low in nephritis with edema (nephrosis).
Albumin (serum) per cent	3.5 to 5.6 Gm.	Muscular activity. Low in nephrosis.
Globulin (serum) per cent	1.5 to 3.2 Gm.	Syphilis, pneumonia, uremia, anaphylaxis.
Fibrinogen (plasma) per cent	0.2 to 0.4 Gm.	Pneumonia, septicemia. Low in liver diseases and cachexias.
Hemoglobin per cent (Haden)	15.6 Gm.	Polycythemia. Low in primary and secondary anemias, chlorosis.
Total nitrogen, per cent	3.0—3.7	Varies chiefly with proteins (albumin, globulin, hemoglobin).
Nonprotein N	25—40	Nephritis, eclampsia, etc. See Urea N.
Urea N	10—15	Chronic and acute nephritis, metallic poisoning, cardiac failure, intestinal or prostatic obstruction, some infectious diseases. Relatively low in nephrosis.
Uric acid	2—3.5	Nephritis, gout, arthritis, eclampsia.
Creatinine	1—2	Nephritis.
Creatine	5—6	Terminal nephritis.
Amino acid N	2.3—3.74	Leukemia, acute yellow atrophy of the liver, severe nephritis.
Ammonia N	0.1—0.2	Terminal interstitial nephritis.
Undetermined N	4—18	Eclampsia.
Glucose	80—120	Diabetes, pregnancy, severe nephritis.
Total fatty acids	290—420	Diabetes, nephritis.
Cholesterol	150—300	Diabetes, nephritis, nephrosis, biliary obstruction, pregnancy. Low in pernicious anemia.
Lipoid phosphorus (lecithin)	12—14	Diabetes, nephritis, pregnancy. In anemia, low in plasma, high in cells.
Total acetone bodies (as acetone)	1.3—2.6	Diabetes.
Acetone + aceto-acetic acid (as acetone)	0.3—2.0	Diabetes.
β -Hydroxybutyric acid (as acetone)	0.5—3.0	Diabetes.
Bilirubin	0.1—0.25	Biliary obstruction, hemolytic anemias. Low in secondary anemia.
CO ₂ capacity (plasma) vol. per cent	55—75 ²	Respiratory diseases, tetany. Low in diabetes, nephritis.
CO ₂ content (arterial blood) vol. per cent	45—55 ²	Respiratory diseases, tetany. Low in diabetes, nephritis.
CO ₂ content (venous blood) vol. per cent	50—60 ²	Respiratory diseases, tetany. Low in diabetes, nephritis.
O ₂ capacity vol. per cent	16—24 ²	Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia.
O ₂ content (arterial blood) vol. per cent	15—23 ²	Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia.
O ₂ content (venous blood) vol. per cent	10—18 ²	Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia.
Lactic acid	5—20	Exercise, eclampsia.
Phenols	1—2	Intestinal obstruction.
Chlorides as NaCl (whole blood)	450—500	Nephritis, cardiac conditions, prostatic obstruction, eclampsia, anemia. Low in diabetes, fever and pneumonia.
Chlorides as NaCl (plasma)	570—620	Nephritis.
Sulphates, inorganic as S	1.04 \pm 0.05	Nephritis. Low in rickets. Normal values 1-2 mg. higher in children.
Phosphorus, inorganic (plasma)	3.7—5	
Calcium (serum)	9—11	Low in infantile tetany, severe nephritis, parathyroidectomy.
Magnesium (serum)	1—3	No changes noted in disease.
Sodium (serum)	315 to 340	Low in cases of alkali deficit.
Potassium (serum)	16—22	Uremia, eclampsia.

*To convert to meq./L., use the formula on page 28.

¹Figures express concentration in mg. per 100 c.c. of whole blood unless otherwise indicated in first column.²Figures represent weighted averages of the observations of several investigators.

TABLE 10—BLOOD CHEMISTRY

(Mg. per 100 c.c. unless otherwise specified)

DISEASE	NONPRO- TEIN NITROGEN	UREA NITROGEN	URIC ACID	CREAT- ININE	GLUCOSE (SUGAR)	SODIUM†	POTAS- SIUM‡	CA- CIUM§
Normal Blood Values	25-40	10-15	2-3.5	1-2	80-120	315-340	16-22	9-10
Acidosis, nondiabetic					Dec.			Inc.
Addison's disease	Inc.	Inc.			Dec.	Dec.	Inc.	
Anemia, Hb., 50% or less	33	18	3	1.2	91	241	107	7.5
Anemia, pernicious					Dec.			N
Arteriosclerosis			3-5		N-Inc.			N
Bichloride poisoning	350-370	To 300	To 16	To 3.5	120-200			
Cardiac decompensation			Inc.					
Cholelithiasis								
Diabetic coma	Inc.	15-30		1-4	300-1200	Dec.	Dec.	
Diabetes, moderate					150-300		Dec.	
Diabetes, renal					80-120			
Eclampsia	To 45	To 25	Inc.	To 2.5	N-Inc.	Dec.		
Gout			4-10					
Hyperparathyroidism	N-Inc.	N-Inc.						To
Hyperthyroidism	N-Inc.	N-Inc.		Inc.	Inc.			N-D
Hypoparathyroidism								To
Hypothyroidism					Dec.			N-D
Intestinal obstruction	To 300	Inc.	N	N		Dec.	Inc.	De
Jaundice, obstructive					N-Inc.			N-D
Lead poisoning								
Liver, acute yellow atrophy	Inc.	Dec.			Dec.			
Liver, cirrhosis					N-Dec.			
Mercurial poisoning	39-158							
Nephritis, acute	40-350	20-200	3-15	2-10	120-180			
Nephritis, subacute with edema	35-200	18-100	5-27	2-10	120-240	Var.	N	De
Nephritis, subacute without edema	30-70	15-35	5-12	2-3.5	120-150			
Nephritis, chronic interstitial	90-350	To 250	To 10	To 16	Inc.			N-D
Osteitis deformans (Paget's disease)	12-42	10-16	2.8-3.8		65-105			9-10
Pneumonia	29-90	12-50	3.6-5.3	1.2-1.8		Dec.		
Pregnancy	Dec.	Dec.		Inc.				De
Prostatic obstruction	30-80	12-40	3-9	1.5-3.5	110-160			
Rheumatism and arthritis			N		N-Inc.			N
Rickets								N-D
Tetany								N
Uremia	90-350	To 600	Inc.	Inc.	Inc.	To 128	N	De

N = normal; Inc. = increase; Dec. = decrease; Var. = variable.

*The values in this table simply indicate those frequently found in certain pathologic states should not be accepted as an absolute basis for differential diagnosis.

†Compiled from the literature.

‡To convert to meq./L., use the formula on page 28.

§Whole blood.

Probably the most important constituent for general day-to-day work is the concentration of nonprotein nitrogen. This amounts to 25 to 40 mg. per 100 c.c. of blood in health. Urea constitutes about 50 per cent of this figure, uric acid about 2 per cent, ammonia 0.3 per cent, and amino acids and the "undetermined nitrogen" fraction, about 46 per cent. In fatal cases of nephritis analyses of blood have shown as much as 400 mg. of NPN (nonprotein nitrogen) per 100 c.c. of blood.

The normal figures for these constituents are as follows:

Nonprotein nitrogen	25-40 mg. per 100 c.c. blood
Urea nitrogen	10-15 mg. per 100 c.c. blood

S* IN HEALTH AND DISEASE†

	CHLO- RIDE AS NaCl§	CHOLES- TEROL	FATTY ACIDS	BILI- RUBIN	PHOS- PHATASE (BODAN- SKY UNITS)	CO ₂ VOL., %	TOTAL PROTEIN, %	ALBU- MIN	GLOBU- LIN	HEMO- GLOBIN GM.,%
5	450-500	150-300	290-420	0.1-0.8	Adults 1.5-4.0 Children 1.5-13.0	Adults 80-50 Children 50-40 28-10 Inc. 63	6-8 Inc. Inc. 15.2	3.5-5.6	1.5-3.2	Men 14-17 Women 13-16
	N-Dec. Dec. 323 N N 480-500	Inc. Inc. To 70 N N-350	Inc.	Dec. Inc.		To 25	4.2-5.3 4.9	Inc. Dec.		7.8 Dec.
	Dec.	13-30 200-800 Inc.	Inc.	N-Inc.	Inc.	To 10 50-30	5.5			
	Dec.			N-Inc.		To 30			Dec.	
	Dec.	To 80	Dec.		Inc.					
	N-Dec. Dec.	To 1350 Dec. Inc.	Inc. Inc.	Inc. N-Inc. Inc. N-Inc.	Dec. Dec. Inc. Inc.	To 120	To 11	Inc.		Dec. Inc.
		N-Dec.			Inc.		Dec. 4.8	Dec. Dec.	N-Dec.	
	N-Inc. 460	Inc. To 900	Inc.			60-20 60-20	Dec. To 3.5	Dec. To 1.0	N Var.	Dec.
20	Var.	Var.	Inc.	N-Dec.	Inc.	N-Dec.				Dec.
4.5					2.6-35.6					
	350-470	Dec. Inc.	Inc.		Inc.	55-32.8 Dec.	5.2-5.3 Dec.	Dec.	Inc.	
		N			N Inc.					
20	Var.	N-Dec.	Inc.			To 12				Dec.

Uric acid	2-3.5 mg. per 100 c.c. blood
Creatinine	1-2 mg. per 100 c.c. blood
Creatine	5-6 mg. per 100 c.c. blood
Sugar	0.08-0.12%
Chlorides as sodium chloride	0.45-0.5 %
Cholesterol	0.15-0.30%

Total nitrogen is eliminated in the proportion of about 15 grams daily. It leaves the body as follows:

Urea (grams)	25.0 (12 gm. N)	or 85%
Creatinine	1.5	or 5%
Uric acid	0.5	or 2%
Ammonia	0.5	or 3%
Rest nitrogen	0.5	or 5%

Source of Urea.—In digestion, protein matter is broken down into amino acids which are picked up by the blood just as pieces of metal are picked up by

a magnet; some are retained and others are transformed into ammonia and eliminated. The greater part of the nitrogen that is eliminated is exogenous and occurs in the form of urea. The blood holds up the carbonates and preserves its neutrality by this means, by eliminating the acids. The greater part of the acids in urine are made up of acid phosphates, derived from the blood. When the blood is no longer able to remove its acids, it calls upon its ammonia for help. (Refer to pages 409 and 411 ff. for discussion of acidosis.) The determining factor is the neutrality of the blood. If enough alkali is administered, the nitrogen entirely disappears.

The **source of creatinine** is entirely endogenous and it is constant day by day in the body. Creatine is methyl-guanidine-acetic acid. It occurs in greatest concentration in striated muscle, in heart muscle, in testes, liver, and kidneys. A smaller amount is present in the brain. A small amount in low concentration appears in the blood and it is constant in amount in health. It is a substance which serves a useful function in the muscles. It is not a waste product of metabolism. If creatine is administered to an animal the major portion cannot be recovered in the excreta.

Creatinine is the internal anhydride of creatine. It is regularly distributed in low concentration throughout the water of the body and is regularly found in the urine. It has been suggested that creatinine is the end product of creatine metabolism. The importance of creatinine in routine blood chemical analysis in connection with chronic nephritis has been very well established. It is generally believed that creatinine is filtered through the glomeruli and concentrated in the tubules without being either reabsorbed or supplemented by secretion. The gravity of a high blood creatinine has been amply established. In chronic nephritis when the blood attains a figure of above 5 mg. per cent creatinine, with a nonprotein nitrogen above 100 mg. per cent, or a urea nitrogen above 80 mg. per cent, one is warranted in making a grave prognosis. It is worth while to note, however, in acute nephritis and after poisons, such as mercuric chloride, where the kidneys are badly injured, the blood creatinine has less prognostic significance.

Creatinuria occurs in conditions affecting the general musculature; for instance, in muscular dystrophy, the total creatinine coefficient rises far above normal. The nutritional dystrophy caused by vitamin E deficiency in rats and rabbits is accompanied by creatinuria. Creatinuria also occurs in certain endocrine disorders, for instance, hyperthyroidism. In infants with hypothyroidism the normal creatinuria is greatly diminished or absent, but can be restored by the administration of thyroid. Creatinuria has been reported in acromegaly and in other diseases of the anterior lobe of the pituitary gland. Castration provokes creatinuria. This can be eliminated by the administration of male and female sex hormones. Creatinuria also occurs in wasting diseases, in some infectious diseases, liver diseases, heart failure, and mental diseases and disorders.

Uric acid is both exogenous and endogenous; about one-half of it is derived from the metabolism of food and one-half from metabolism of one's own tissues. If liver is eaten, the amount of uric acid present can be raised. The purine bases, adenine and guanine, which have been released with the decomposition

of nucleic acid, are amino purines. Certain enzymes contained in the tissues convert these amino purines to hypoxanthine and xanthine and then to uric acid. Uric acid is oxidized by the enzyme uricase to form allantoin.

Uric acid is a difficult substance to dissolve. It is soluble 1 part in 39 of pure water. Urates are soluble in 1 part in 500 under conditions as they exist in the body. Uric acid is the most difficult for the kidney to excrete of the nonprotein blood constituents; urea comes next, and creatinine last. Expressed in other terms, creatinine is the easiest constituent for the kidneys to eliminate, urea is the next, and uric acid is the last to be eliminated. Again, urea exists in the body in twenty times as high concentration as creatinine and it therefore takes twenty times as much work for the kidney to eliminate its urea as its creatinine.

ANTICOAGULANTS OF BLOOD IN BLOOD CHEMICAL ANALYSES

Anticoagulants are agents used to prevent coagulation and, in some cases, to prevent the deterioration of blood samples intended for blood chemical determinations. Only those anticoagulants should be selected which will in no way interfere with certain chemical methods.

Various anticoagulants have been suggested for blood chemical procedures. The best is lithium oxalate. It is more soluble than sodium or potassium oxalate and prevents a white precipitate in uric acid determinations. Potassium oxalate alters the electrolyte distribution in blood, interferes with precipitation of protein in the Folin-Wu method, and gives too low sugar values. It may cause clouding when nesslerizing. The same objections hold true for sodium oxalate.

Sodium fluoride is used by some laboratories in micro-sugar determinations. It will preserve the blood for 24 hours at room temperature, and 5 to 6 days at refrigerator temperatures. However, it interferes with the action of urease in urea determinations. Lithium citrate, used as a powder in 30 mg. quantity, is impractical for routine use, and is employed only when determinations of mineral constituents of whole blood are desired.

Heparin is expensive and the dry powder is not readily soluble in blood.

Ammonium and potassium oxalate mixture is used by some workers for hematologic procedures, especially in blood grouping tests. It is not recommended for blood chemical procedures; particularly it must not be used when testing for nitrogenous constituents. The formula is given on page 567.

1. Potassium or Sodium Oxalate.

Preferably use 20 mg. of potassium or sodium oxalate, reagent grade, for each 10 c.c. of blood. Prepare this potassium or sodium oxalate as follows in the bottle to be used for collection of the blood. Pipette 0.1 or 0.2 c.c. of a 10 per cent aqueous solution of neutral potassium oxalate, or 0.7 c.c. of a 3 per cent aqueous solution of ammonium or sodium oxalate, for each 10 c.c. of blood to be collected, in the bottle (a wide mouth bottle); dry in the oven at not over 80° C., after stoppering with a cork.

2. Heparin. Use 1 mg. for each 100 c.c. of blood.

3. Potassium or Sodium Fluoride.

Use 60 mg. of potassium or sodium fluoride, reagent grade, for each 10 c.c. of blood, as a preservative. Fluorides are not to be used if chloride or urea by the urease method is to be determined.

4. Sodium Citrate.

Use 200 mg. of sodium citrate for each 10 c.c. of blood.

5. Sodium Fluoride Compound.

Mix sodium fluoride, reagent grade, 10 gm., thymol, 1.0 gm., and 2 gm. of lithium oxalate, by grinding. Use 35 mg. of this mixture for each 10 c.c. of blood.

6. Lithium Oxalate.

Use 0.5 c.c. of a 1 per cent solution of lithium oxalate for 5 c.c. of blood. Dry in an oven at about 80° C. This is a very good anticoagulant for NPN determination.

MANNER OF PROCURING AND HANDLING BLOOD FOR CHEMICAL EXAMINATIONS

The blood is always taken early in the morning before the patient has eaten. Since the established normal figures were obtained under these conditions, strict adherence to these rules is necessarily the only means of making accurate comparisons.

The tests must be made as soon after withdrawal of the blood as possible. If it is impossible to proceed to the end of the test, precipitate the proteins at once. The blood may then stand until one has time to complete the test. If blood is to be sent away, add a drop of concentrated sulphuric acid to the contents as a preservative.

Instruct the patient to report to the laboratory without breakfast. Have on hand prepared bottles of anticoagulant. Use one of the bottles described under **Anticoagulants** (above), preferably the lithium oxalate bottle.

Have ready a tourniquet; one of the specially prepared bottles; a sterile syringe and needle, 21 gauge, 1½ inch; sterile sponges; alcohol and iodine; adhesive tape.

Prepare the patient in the manner described on page 2031. Insert the needle into the vein, and withdraw the blood into the syringe; then eject it into the bottle containing the oxalate. Withdraw from 10 to 25 c.c., depending on the tests to be made. Be sure to take enough blood. It is better to take a few c.c. too much than not enough. Remove the tourniquet, disinfect the skin, and apply pressure to the puncture; then place a piece of adhesive tape over the puncture. Replace the cork in the bottle, and invert about 20 times until the blood is thoroughly oxalated. It will be seen to turn from a dark to a light red.

PREPARATION OF GLASSWARE FOR BLOOD CHEMICAL ANALYSES

Glassware must be chemically clean and dry. This means all volumetric glassware, such as pipettes, burets, and flasks, must be cleaned with cleaning solution. To clean glassware, rinse thoroughly in tap water. Then immerse in a cleaning solution; rinse thoroughly to remove the cleaning solution. Rinse with distilled water, and allow to drain dry, or hasten drying in an oven.

Potassium Dichromate-Sulphuric Acid Cleaning Solution

Distilled water	500 parts
Technical potassium dichromate	200 parts
Technical sulphuric acid, conc.	500 parts

Add the potassium dichromate to the water in a Pyrex Erlenmeyer flask. Add the sulphuric acid to this solution, a small amount at a time, cooling the flask under running water until all the sulphuric acid has been added. Keep the cleaning solution in a crock in the laboratory.

Remove all traces of the acid from the glassware, after immersion, by rinsing several times in hot tap water. Rinse in distilled water, and dry in a dry heat sterilizer. When cleaning pipettes, be sure the acid touches the inner surface as well as the outer surface.

Henry and Smith¹ conducted a series of experiments using potassium or sodium dichromate for cleaning glassware and found that even after repeated washings a large amount of dichromate still remained on the glassware. The effect of dichromate on growth of two strains of *Staphylococcus aureus* was followed turbidimetrically and checked by duplicate pour-plate dilution counts. This amount was toxic to growth. Because of the extreme difficulty in ridding glassware of dichromate after cleaning in "cleaning solution" and its great toxicity for living cells and enzymes, it is believed highly advisable in laboratories dealing with such material to clean all glassware by another method, such as 10 per cent nitric acid, a detergent, or 1 to 5 per cent trisodium phosphate.

Nitric and Sulphuric Acid Colorless Cleaning Mixture

The potassium dichromate-sulphuric acid mixture above described is an excellent cleaning solution. Its disadvantages are that it becomes rapidly spent with use, turning green because of the reduction of the chromium trioxide by organic materials on the glassware. Furthermore, this mixture has a dark color, making it difficult to find small objects and remove them from the bath. It is claimed by Laug² that traces of chromium which adhere to the glass can be removed only by very extensive washing and, therefore, may contaminate biologic materials which subsequently come in contact with the glass.

For this reason, the following mixture of concentrated sulphuric and nitric acid has been recommended by Tobie.³ Maintain a bath of sulphuric acid in a large evaporating dish or other acid-resisting container on a gas range or electric hot plate under a hood, and to this add a small amount of nitric acid with thorough stirring at the beginning of each day's work, before turning on the heat. Use a Duriron dish which avoids the danger of breakage. The nitric acid should be well stirred into the cold sulphuric acid, otherwise it will form a layer on the surface and will evaporate rapidly as the bath becomes hot. This cleaning bath may be maintained for several months. It should be discarded only when the sulphuric acid becomes viscid by the slow accumulation of inorganic salts from the oxidized materials.

Keep this in a hood with a good current of air to avoid breathing irritating fumes. Avoid splashing the mixture on the hands or clothes to prevent burns. Remove glassware from bath with steel crucible tongs and keep under the hood to cool before rinsing with water.

Do not heat this mixture too hot because white fumes of sulphuric acid come off, causing a large loss of acid.

Wetting Agents for Cleaning Glassware

There are a number of wetting agents now on the market for cleaning glassware. Our personal experience leans to Haemo-Sol for blood chemical work. It is a product of Meinelke and Co., Inc.⁴ It is very effective in repeated use and will not etch glass. It

¹Henry, R. J., and Smith, E. C.: Science **104**: 2705, Nov. 1, 1946.

²Laug, E. P.: Indust. & Engin. Chem., Anal. Ed. **6**: 111, 1934.

³Tobie, W. C.: J. Lab. & Clin. Med. **26**: 1797, 1941.

⁴Meinelke and Co., Inc., 224 Varick St., New York 14, N. Y.

strips tubes and vessels of proteins, oils, fats, and other material which commonly soil glassware. It leaves the laboratory glassware chemically pure and absolutely clean. Haemo-Sol is completely rinsable and completely soluble. It has low surface tension and controlled pH. Because it leaves no residue, it is especially valuable in the most exacting titrations and tests.

Another good wetting agent is Alconox.² It is neither soap nor strong alkali, and is efficient in water of any degree of hardness.

Aerosol³ is also a good wetting agent and cleanser. For use, 1 teaspoonful of Aerosol 10% is added to a gallon of water.

Do not use detergents to clean glassware that is to be used for prothrombin time determinations.

Care of Burets

Solutions must not be left standing in burets. The buret should be drained and covered, or inverted if it is to be used shortly. Do not allow alkali to remain in the buret as the stopcock may freeze, and glass will be etched. Be sure the stopcock is removed from the buret, but attach it by means of a rubber band when it is not in use.

STANDARDIZATION OF REAGENTS

Reagents used in blood and urine chemical analysis should be standardized against known solutions. If standardization is impossible by titration methods, then it is accomplished by color comparative methods. To standardize a solution, develop the color of the standard for the colorimeter according to the technic used in the test which employs this reagent, using reagents of known strength. At the same time, develop a similar standard, using the same reagents, substituting, of course, the reagent to be tested. Only one reagent used in this process can be unknown. Match the colors in the colorimeter. They should match at the same height on the scale. If they do not match, either dilute the reagent according to calculations, or make it stronger. It is better, if it is not an expensive reagent, to remake it. If a solution is diluted accurately in a volumetric flask, if all chemicals are weighed accurately on an analytical balance, and no substance is lost in transferring, the solutions should be accurate, and the testing just a means of checking.

College of American Pathologists Standardized Solutions

A distinct aid has been given laboratory workers through the agency of the College of American Pathologists. This organization has developed a project to increase the efficiency of laboratory determinations by the furnishing of small volumes of reassayed, highly standardized, analytical solutions in hard glass ampules. These reagents are sold by this organization to laboratories for the purpose of checking their own solutions. The ampules are hermetically sealed until opened for use, and cannot be resealed without destroying the analytical values of the solution. This obviates the errors due to storage of solutions in stoppered or screw-capped containers where contamination, concentration through evaporation, dilution through use of wet pipettes, etc., interferes with accuracy in chemical procedures.

²Standard Scientific Supply Corporation, 34 West Fourth St., New York 12, N. Y.

³Fisher Scientific Co., 711 Forbes St., Pittsburgh, Pa.

With these ampules, after the desired volume has been removed, the open ampule should not be kept longer than the balance of the day before the remaining solution is discarded.

These ampuled solutions can be used to test technical ability by issuing the ampule (with or without the pasted label) and cautioning the personnel not to use protein-precipitating agents as a diluent. In this case, specify that ammonia-free distilled water should be used. Each step in the routine technic should be followed except for the choice of diluent to prepare a "blood filtrate." The same volumes should be used as specified in each step of the routine technic. An identical procedure could be followed in developing a known positive control as a standard for each day. A larger volume (50 c.c. diluted standard used as a substitute blood filtrate) would offer greater opportunity to establish a correction factor for the laboratory stock solution or to develop practice sessions designed to improve technic.

This controlling agency offers the following precautions about this type of work. These solutions are for analytical purposes only, and should be used only in clinical laboratory chemical determinations. They should not be used in the animal or human body. Preservatives have been added where necessary. Only chemically clean, dry glassware should be used in connection with the analytical use of these solutions. In case the opened ampule will not admit a volumetric pipette, the opening can be enlarged by prying with a clean, dry, blunt instrument, or the solution can be poured into a chemically clean, dry beaker. Diluted solutions may deteriorate and should be discarded.

At the time of this publication, the College of American Pathologists is prepared to furnish the following solutions:

<i>Solution</i>	<i>Strength</i>
Glucose	1 ml. = 2 mg.
Nitrogen	5 ml. = 1 mg. N
Chloride	1 ml. = 5.84 mg. NaCl
Calcium	1 ml. = 0.1 mg. Ca
Uric acid	1 ml. = 1 mg.
Creatinine	1 ml. = 1 mg.
Phosphate	5 ml. = 0.4 mg. P
Flame photometry	Na 140 meq./liter, K 4 meq./liter
Sodium and potassium	

All these solutions are furnished in 5 ml. ampules with 6 ampules per box.*

CLINICAL COLORIMETRY¹

Color reactions of liquids have, for a long time, played an important part in chemical analysis. A clear liquid appears colored if it transmits light rays of some wave lengths less than of other wave lengths (selective absorption). This nonuniform absorption of the various parts of the spectrum is sometimes caused by very minute quantities of a certain substance in a solution, thereby making color reactions one of the most sensitive tools of chemical analysis.

*These are available by ordering from the College of American Pathologists, 203 North Wabash Ave., Chicago 1, Ill.

¹Loewenberg, F.: Lab. Digest 9: 1, June, 1945. (Elaboration of this material can be found in the Photovolt Reference Book, Photovolt Corporation, 95 Madison Ave., New York 16, N. Y.)

In taking advantage of color reactions for quantitative analytical purposes, a first refinement consisted in using the depth of the color to draw conclusions regarding the quantity of the substance causing the color. Such conclusions are justified, once it has been established (1) that the depth of color is a definite measure of the quantity of the substance, (2) that no other substance which may be present can interfere by causing a similar color reaction. If this has been ascertained, the color reaction can serve as a basis for quantitative analysis.

The simplest method of carrying out quantitative colorimetric tests is to make up solutions containing known quantities of the substance and to compare the color of the unknown solution visually with the color of the knowns. The unknown will then appear equal to one of the known or will lie between two of them. In this way, it is entirely possible to carry out quantitative colorimetric tests without any instruments and without special equipment.

Artificial Color Standards

The foregoing method of colorimetric analysis makes it necessary to prepare a whole series of known solutions each time. Generally, the depth of color of the known solutions (standards) cannot be relied upon to remain stable, one of the main inherent characteristics of color phenomena, surface colors as well as liquid colors, being their tendency to change (fade). However, artificial color standards have been developed which, while being completely different in their chemical nature, are sufficiently similar to the samples as to visual color appearance to permit grading of the unknown. Being selected mainly from the point of view of best possible stability, they are sufficiently permanent for many practical applications and have found extensive use, in the shape of color-comparison sets, for all kinds of colorimetric tests.

In some of the visual comparison tests by means of artificial standards, it is possible to get along with one rather than with a series of color standards. The specimen is then diluted in small steps until its color appearance matches the artificial standard, and the result is derived from the amount of diluent required to produce the match. This method is employed, for instance, in the visual hemoglobinometers of the Sahli type. The method does away with some of the inherent inaccuracy of color comparison sets which, in order to cover a sufficiently wide range, must be made up in comparatively coarse steps. However, the color matching by progressive diluting, while being applicable to such simple tests as hemoglobin, is not possible in those colorimetric determinations which require the color to be developed by means of reagents.

A limitation common to both methods employing artificial color standards lies in the fact that the results depend somewhat upon suitable light being available. Since the artificial color standards are in no way related to the unknowns chemically and since they may differ widely in their spectral transmittance, it is impossible to obtain the same matching point under all kinds of illumination. What appears as a color match in daylight may not appear matched in incandescent light and vice versa.

Duboscq Type Colorimeters

In the methods described above, the artificial color standards and the glass tube containing the unknown are usually inserted into frames and held against

the light for the purpose of visual comparison. As a result, dark dividing strips appear between the colors to be matched. Now, the capacity of the human eye to judge a color match is seriously impaired by this dark strip and is by far not as high as when the two colors appear next to each other without even a thin dividing line in between.

It was, therefore, a great step ahead when the Duboseq colorimeter was developed in which the colors to be matched appear in a split field without a dividing line. In these visual colorimeters, just as in color matching by progressive diluting, the eye of the operator is used merely as a "zero indicator," i.e., it is called upon only to judge when the two fields appear matched. The human eye is much better suited for judging a match than for estimating the magnitude of color differences. Therefore, the sensitivity obtainable with the Dubosq is far higher than the one which can be achieved by means of color comparison sets.

In Duboseq colorimeters, the color match is produced by adjusting the light path in the liquid rather than by progressive diluting. The instrument comprises two cups with glass plungers reaching into them. One cup is filled with a solution of known concentration, the other with the unknown. By adjusting the position of the plungers, a match can be obtained in the split field. Then, making use of two laws of physics, Lambert's law and Beer's law, a conclusion can be drawn from the position of the plungers regarding the concentration of the unknown solution in relation to the concentration of the known.

Since the two solutions are similar chemically as well as spectrally, the results obtained on the Duboseq are comparatively free of error due to light sources. However, the results remain dependent upon individual judgment and are frequently not reproducible from one operator to the other due to differences in color perception. It is inherent to colorimetry with a Duboseq-type instrument that a known solution must always be run along with the unknown. In order to obtain accurate results with the Duboseq, it is necessary that the known solution have a concentration close to the unknown. If the two concentrations differ widely, the Duboseq (the measuring principle of which is based on Beer's law) will often give an appreciable error due to the fact that the law is not always applicable over a wide range. For this reason and since the operator can frequently not estimate the concentration of the unknown beforehand, he often must run several known solutions rather than one along with the unknown.

Colorimeters are expensive instruments and must be handled carefully. Chemicals and dust ruin optical surfaces. It is important to clean colorimeter cells thoroughly after use. Paper tissues may be used for drying the plungers and cells. Be sure that the solvents which are used in colorimeters are not put into glass cells, the edges of which are not fused.

The Dubosq Colorimeter

The original Duboseq instrument is made in France. Duboseq colorimeters are manufactured in this country by American Optical Co., Scientific Instrument Division, of Buffalo, N. Y., Bausch & Lomb Co., of Rochester, N. Y., and E. Leitz & Co., in exact accordance with the original French pattern and are guaranteed to offer identical results. Klett Manufacturing Co., New York, also makes a Duboseq type colorimeter.

The mirror supported by the base of the instrument has two surfaces, one clear for direct reflected light, the other opaque for diffused light.



Fig. 78.

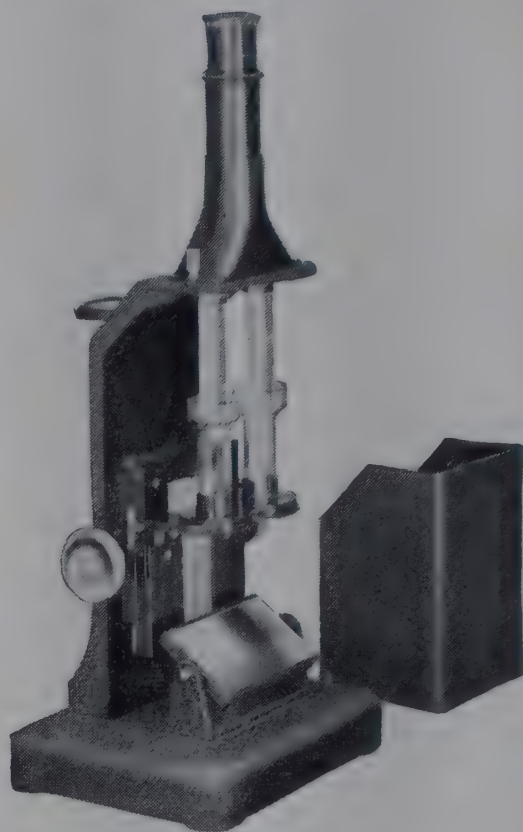


Fig. 79.

Fig. 78.—Spencer Duboscq type colorimeter. (American Optical Company, Scientific Instrument Division.)

Fig. 79.—Duboscq colorimeter. (Bausch and Lomb Co.)



Fig. 80.—Filling a colorimeter cup.

The liquids to be compared, viz., “Known” and “Unknown,” are placed in the two glass cups, the bottoms of which consist of plano-parallel glass plates. So as to vary at will the thickness of the two columns of liquids, through which the light passes, two glass plungers are provided, which reach into the glass cylinders. These plungers are of solid glass, their upper and lower surfaces being plano-parallel. They are moved along their perpendicu-

lar axes by a rack and pinion motion, and their lower surface can be brought into contact with the glass bottom of the cylinders at the zero point of the scale.

This scale consists of a graduation in millimeters and a vernier 10 mm.: 10, which permits measuring with precision the extent of the displacement of the cylinders. Two cemented prisms, in housing, are mounted above the



Fig. 81.—Setting the colorimeter standard.



Fig. 82.—Colorimeter fields.

two plungers which receive the pencils of light coming from the plungers and bring these two pencils of rays in contact by two interior reflections. These are observed through the eyepiece situated above the prism housing. The colorimeter can be illuminated either by daylight, which is preferable, or by artificial light, using blue glass.

Directions for Using the Duboscq.—One of the glass cylinders contains the standard solution called the “Known” and the other the liquid to be studied called the “Un-

known." Any desired thickness of the solution can be viewed between the bottom of the cylinders and the base of the plungers by moving the cylinders within their perpendicular axes, using the rack and pinion. For colorimetric comparison, first regulate the mirror by looking through the eyepiece, focus the latter by its mounting, turn the mirror so that the two halves of the field of vision appear of equal intensity. To accomplish this properly, the cups must be empty and completely clean. Then pour the solutions into the cylinders. The cylinder containing the known solution or standard is then lowered so as to obtain a specific thickness of this solution between the bottom of the cylinder and the base of the plunger. The half of the field of vision representing the standard solution will become darker, while the color in the other cylinder holding the unknown will appear of a different color. By raising or lowering the cylinder of the unknown, the two halves of the field can be easily brought to an identical intensity. When this is accomplished, it is then only necessary to read on the scale the heights of the two layers of liquid, possessing an equal power of absorption. The difference between the two scale readings, controlling the known and the unknown solutions, represents the coloring matter contained in the unknown proportional to the coloring matter as it is contained in the known.

Klett Colorimeter

Assembling the Klett Colorimeter.—To attach the instrument to the lamp house which is packed separately, put in the two screws on the top of the instrument in the two corresponding slots of the lamp house first. Do not tighten. Connect the instrument to the base which carries the switch, and when all is in proper position, tighten it.

To get zero point put in the cups, run up stage until bottom of plunger touches bottom of cup, then loosen vernier, bring upper line of vernier in line with first line of scale; tighten nut again.

If the "side by side" field does not appear to be even, slowly turn the ground mirror reflectors. These reflectors work independently of each other, and the reflected light can be adjusted so as to insure a very even field.

The dividing line in the field should appear as distinct and as thin as possible. For adjustment, move the upper telescope tube up or down.

When using daylight be sure of good lighting conditions. Obstructions, such as large trees and buildings in front of your windows, will prevent an even illumination on both sides of the field. While this, however, can be more or less corrected through the manipulation of the ground mirror reflectors, the most suitable position giving good, even light should be selected.

More accurate comparison of colors will be obtained by resting the eyes at short intervals, for instance, by closing them or by looking away every two seconds for a period of two seconds.

Directions for Keeping Klett Colorimeter in Proper Condition.—Keep reflector clean; no accurate readings are obtainable if mirrors are soiled; do not allow any moisture to remain on reflector.

If long spindle threads should become loose, loosen small set screw and regulate pivot; release screw until dead motion disappears; then tighten screw again.

Keep threads free from dust. If the black varnish of the instrument is dusty, it may be cleaned with any fine oil.

Cleaning of mirrors may after a time cause the ground surface to become somewhat brighter, particularly on the one side used and cleaned most frequently. Therefore clean both mirrors equally, but if a difference should occur rub the darker side with a moist tuft of cotton containing a little "Bon Ami" powder. Attention of this sort keeps the mirrors in good condition.

The prisms are enclosed in a dust-proof housing and they should be removed only when absolutely necessary. Remove outer cover plate by using screw driver and unscrew the prism plate the same way; it can then be taken out.

To clean the lenses of the telescope, it is necessary to separate the whole telescope by unscrewing it from the prism housing. Then pull out the eyepiece, and the lens carrier

can be pushed out with the finger from below through the large diameter tube and lenses cleaned. The whole operation does not require any tool and it is hardly possible to make a mistake when putting it back.

Lamp House.—The lamp house is divided into two parts. The upper part can be removed by unscrewing the set screws on the side of the body. The plain glass is just clamped in position and can easily be removed for cleaning; the condenser lens does not have to be removed and can be cleaned from the inside of the lamp house; also from the instrument side. The plain glass can be taken out if necessary and colored glass put in instead for screening. Should it become necessary to put in a new lamp bulb, care should be taken that the heavy filament support inside the bulb does not face the instrument side to avoid casting shadows in the field. This is accomplished by moving the lamp back and forth and turning it around on its own axis. Once the lamp is set it should be tightened with the set screw and not be touched unless it is absolutely necessary. Care should be



Fig. 83.—Klett colorimeter. (Courtesy A. S. Aloe Co., St. Louis, Mo.)

taken that the daylight screen above the light is always kept clean. The light should be shut off when not in actual use; the switch handle on the lamp socket makes it easy enough to do so. The lamp bulb is especially made for this instrument and has a longer filament than the ordinary commercial lamp of the same dimensions and can also be had for 220-240 volts. The position of the lamp socket can be altered by loosening the set screw in the clamp with a screw driver.

The switch for the lamp is located in the part which connects the instrument proper to the wooden base of the push-button type.

Directions for Using.—Assuming that the mirrors are placed so that the bottoms of the cups are equally illuminated, as shown in the eyepiece, when the cups are used with only distilled water the following procedure is perhaps the simplest and in many cases the most accurate:

Setting of the zero point is very simple: Run cup up until the bottom of the plunger touches the bottom of the cup; then loosen the nickel-plated

screw on the bottom of the scale and move the scale up or down until the zero line of the vernier meets the zero line of the scale accurately.

At the beginning of a series of analyses the inside of both cups and the outside of both plungers of the instrument are first rinsed with distilled water and then with the colored solution to be used as a standard. Then the cups are nearly filled with this solution and placed in position in the instrument. The position of the left-hand cup is adjusted until the scale reading is exactly 20.0; the position of the right-hand cup is then adjusted until both sides are equally illuminated, this being determined as the average of several independent settings. This setting of the right-hand cup, that is, at the average of several settings, is retained for the whole series of comparisons with the standard.

Now the solution of the left-hand cup is discarded and the cup and plunger rinsed with the solution next to be determined and the cup nearly filled with the new solution. It is placed in position in the instrument and the height is adjusted until illuminations on both sides are again equal, when the scale reading is recorded.

The revolving result table is per ratio only and is the result of dividing the known by the unknown. The table for the Newcomer standard is separate, but printed on the same sheet and appears after the regular table is rolled off. On this table the reading is direct.

The artificial illumination should always be used in preference to the daylight reflector; the latter should be in a vertical position to act as a shield for the substage lamp. Should the lamp burn out or some defect in the wiring prevent its use, the daylight reflector should be used until a new lamp is obtained.

The two round recesses in the base on each side of the two uprights are for the purpose of removing the glass standards when not in use. To use one of the standards the left-hand plunger must be removed and the standard inserted in the top of it.

As dust may drop through the small diaphragm in the eyepiece, it is advisable to remove the eyepiece from time to time and clean the lens underneath.

For the benefit of those who are making hemoglobin determinations by the Newcomer method, by a small attachment the Klett colorimeter may be used for this purpose. This attachment is simply a small glass plate inserted at the top of the left-hand plunger of the colorimeter (see page 583).

Formula for Computation With Any Plunger Type of Colorimeter

The following formula may be used to compute any blood chemical test read on the plunger type of colorimeter:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{\text{Dilution of Unknown}}{\text{Dilution of Standard}} \times \text{Strength of Standard in mg.} \times \frac{100}{\text{c.c. Blood Used}} = \text{mg. in 100 c.c. blood.}$$

For example, if the standard is set at 15; the unknown matches at 10; the unknown solution was diluted to 50, and the standard to 100; the standard is made so that it con-

tains 1 mg. in the quantity used in the test; and 2 c.c. of blood were used in the test, then the formula is

$$\frac{15}{10} \times \frac{50}{100} \times 1 \times \frac{100}{2} = 37.5 \text{ mg. in 100 c.c. blood}$$

PHOTOELECTRIC COLORIMETRY¹

Photoelectric Cells.—An entirely new element was introduced into analytical colorimetry when the first suitable photocells became available around 1934. Most of the photocells used in colorimetry are of the barrier-layer type. When struck by light, these cells give off an electric current which, under certain conditions, is proportionate to the amount of light.

Photocells are frequently referred to as "electric eyes." However, aside from the fact that they react to light, they have very little in common with the eye. They are superior to the human eye in some respects and inferior in other respects. At any rate, the photocell does not "see." The only characteristic of the photocell which is made use of in a colorimeter is the proportionate relationship between incident light and electric current delivered.

A photocell is completely "color-blind." Without the use of any additional element, it cannot distinguish between the various parts of the spectrum. However, by combining the photocell with monochromatic light, it can be made to be responsive to certain bands of the spectrum only, and unresponsive to others.

The most important advantage of the photocell is its capacity to evaluate light values over a wide range. The human eye, so far as colors are concerned, is mainly suited to indicate a color match. If two light values differ from each other, the observer is completely unable to tell even approximately how much stronger the one light value is than the other. The same does not apply to the photocell. When a photocell is connected to an electrical indicating instrument, a given light impinging upon the cell may cause a needle deflection which, by means of certain circuit elements, can be made to cover the full range of the meter. If the light is reduced to a fraction of its initial value, this fraction will be accurately indicated by the needle deflection. Let us assume that the light is reduced to 50 per cent of its initial value. Then, if the circuit comprising photocell and indicating instrument has linear response, the needle will be accurately on half-scale. For an observer, it would be impossible to tell visually whether the light has 75, 50, or 25 per cent of its initial value.

Even when using a photocell, colorimetry remains a comparison of light values. However, in view of the characteristics of the cell, it is no longer required that the two light values to be compared be equal or even approximately equal. This makes it possible to refer all light measurements to one liquid standard which is always freely available, namely, distilled water. Since distilled water has practically no absorption, the transmission value of 100 per cent is assigned to it, and the light transmission of any other solution can be expressed as a percentage of distilled water. It is no longer required to grade the unknowns by means of artificial color standards or to produce a match by pro-

¹Loewenberg, F.: Lab. Digest 9: 1, June, 1945. (For full particulars regarding the Lumetron photoelectric colorimeter consult the Photovolt Reference Book, Photovolt Corporation, 95 Madison Ave., New York 16, N. Y.)

gressive dilution or by varying the length of the light path in the liquid. The results are not dependent upon a suitable light source or upon the individual judgment of the operator. The needle position is a definite indication of the light transmission of the liquid under test.

Sensitivity.—Photoelectric colorimetry has found extensive application in most fields of chemical analysis. Blood chemistry is a particularly suitable field of application, because the quantity of specimen available is always very limited and the quantity of substance to be determined in the specimen is usually extremely small. Photoelectric colorimetry permits the measurements of quantities which could not be measured by gravimetric or similar methods.

In the blood sugar test, for instance (using the Lumetron Photovolt), a blue color is obtained by treating the blood filtrate with copper tartrate and phosphomolybdic acid. If a sugar solution containing as little as 0.01 mg. per 100 c.c. is subjected to this treatment and if the depth of the blue color is measured on a photoelectric colorimeter, using a red filter, a clearly readable deflection would be obtained. In this instance, photoelectric colorimetry makes it possible to detect the presence of one part sugar in ten million parts of water. This test requires only 2 c.c. of the original sugar solution so that, actually, the presence of a quantity as small as 0.0002 mg., or two-tenths of one microgram, is being detected. Not all the clinical colorimetric methods are of such extremely high sensitivity.

Monochromatic Light Source.—While all these visual color-comparison methods are carried out with light that is essentially white and comprises all the wave lengths of the visible spectrum, a photoelectric colorimeter always makes use of monochromatic light produced by a prism, grating, or filter which isolates a certain wave band and suppresses the response of the photocell outside this band. The result is that much finer distinctions are made possible.

In many colorimetric determinations, for instance, a yellow coloration is obtained. When viewing such yellow solutions in ordinary light, the human eye is fairly poor in distinguishing small differences in shade. In a photoelectric colorimeter, yellow substances are mostly measured with blue filter, i.e., a filter which absorbs the red part of the spectrum. Two yellow solutions which appear completely equal to an observer may show wide differences in a photoelectric test. The reason is that the two solutions, while equally transparent to yellow and red light, differ very much in their blue transmission. The eye is "misled" by the high yellow and red transmission of the two solutions. The photocell is kept, by the filter, from being influenced by their yellow and red transmissions and registers only in the blue, in which region the two solutions show a marked difference.

Fleeting Colors.—There are some important clinical colorimetric determinations which cannot be carried out on a visual comparison instrument. This refers to tests in which a fleeting color is being measured as, for instance, in the determination of vitamin A. The coloration in the vitamin A determination appears and fades within fractions of a minute. Obviously, it is not possible to time the standard and the unknown in such a way that the maximum color is developed in both simultaneously.

Blanks.—One main advantage of photoelectric colorimetry is that the readings are mostly taken against distilled water. Nevertheless, there are a number of determinations where the readings are taken against a blank obtained by running a solution of zero concentration through the procedure along with the unknown. The purpose of reading against the blank is to allow for that part of the coloration which is not attributable to the substance to be determined but to the reagents.

Chemistry of Clinical Colorimetric Methods.—In these determinations, the test consists essentially in the photoelectric measurement of an inherent color. This refers particularly to the hemoglobin determination in which the color of the diluted blood specimen serves as an indication of the hemoglobin content (acid hematin and oxyhemoglobin methods). An inherent color is also measured in the icteric index determination. In the PSP test, one measures the color of the urine after a dye has been injected into the blood stream.

While the foregoing determinations consist in the measurement of a pre-existent color, all the other clinical colorimetric determinations include chemical procedures. The two fundamental prerequisites for a colorimetric analysis are (1) the depth of color must be a definite measure of the quantity of the substance to be determined; (2) no other substance which may be present must interfere by causing a similar color reaction. Since the body fluids are extremely complex in their chemical composition, the purpose of the first steps of the chemical procedure is mostly to separate the substance to be determined. Then, in the second part of the procedure, reagents are introduced which cause the color reaction. After having been subjected to this chemical procedure, the specimen is ready to be tested on the colorimeter.

Calibration.—One of the basic requirements of a photoelectric analytical method is that the author must devise a way in which the color readings can be correlated, numerically, to concentration values. The deeper color will mostly indicate a higher concentration. However, it is necessary to know exactly what concentration value corresponds to what depth of color, or, in terms of the instrument, what needle position indicates what concentration.

Obviously, it is not possible to establish such correlations by means of computations alone. The basic law governing the quantitative relation of concentration to depth of color is unknown. Consequently, the correlation must be established empirically, i.e., by actual tests. It is necessary to make up solutions of known concentration, to subject them to the procedure for developing the color, and to read them on the instrument. This is called *calibrating* and represents the only possible way in which photoelectric readings can be translated into concentration values.

Frequently, it is necessary to subject the known solutions not only to the steps for developing the color but also to steps for separation and purification. Generally speaking, the calibrating procedures are laid out so as to insure that the solutions of known concentration, although made up without the aid of blood, yield a depth of color which is identical with the depth obtained in an actual specimen of the same concentration.

In other instances, the substance to be determined is not available in pure form so that it is not possible to make up solutions of known concentration. This applies, for instance, to hemoglobin. It is, then, necessary to resort to in-

direct calibration methods. In the blood iron and hemoglobin method of Wong, for instance, solutions of known iron concentration, rather than of known hemoglobin concentration, are used for calibrating. A colorimetric procedure for iron in blood is applied which, in conjunction with the iron calibration, furnishes the iron content of the unknown. Then a conclusion is drawn regarding the hemoglobin contents of the unknown on the basis of the known amount of iron contained in 1 gram of hemoglobin.

When solutions of known concentration cannot be made, another indirect calibrating method consists in analyzing some available blood sample by way of another method, after which this sample is employed as a "known" for calibrating purposes. In the case of hemoglobin in blood, this latter calibration method is used in calibrating for the acid hematin and oxyhemoglobin tests. The blood iron test is utilized only once for the purpose of analyzing the specimen which then serves as a "known," for calibrating in the acid hematin and oxyhemoglobin procedures. These latter procedures are suited for routine work inasmuch as they represent essentially a measurement of inherent color and require nothing else except diluting of the blood specimen.

While the foregoing detours are necessary for calibrating where solutions of known concentration cannot be made, certain short cuts for calibrating have been devised for other determinations. They consist in making colored solutions which, without any further treatment for color development, can serve as standards for calibrating. The colored solutions, while not related chemically to the substance to be determined, are spectrally similar to the unknowns. This refers, for instance, to the potassium permanganate solution which can be employed as an alternative calibration solution in the bilirubin test. Such substitution of an unrelated substance for solutions of known bilirubin concentration is permissible because a definite correlation has been established between the concentrations of bilirubin solutions and potassium permanganate solutions of equal color. Potassium permanganate thus can serve as substitute standard in calibrating and offers the advantage that it does not require any chemical procedure for color development. In a somewhat similar manner, potassium dichromate is employed as a substitute calibration solution in the icteric index calibration.

Individual Calibrating of Instruments.—Photoelectric colorimeters must, by necessity, be calibrated individually, i.e., every single instrument has to be calibrated separately. If it were possible to manufacture photoelectric colorimeters so that all instruments are completely identical in their response from one to the other, the correlation between needle indication and concentration value could, for each determination, be laid down in printed form by the manufacturer. Such calibration cards could then be furnished with the instrument at practically no expense to either the manufacturer or the user.

Unfortunately, this is not feasible because some of the main component parts of a photoelectric colorimeter are of such nature that they inherently cannot be produced with identical characteristics. Therefore, there is a certain variation in the response of the instruments from one to the other, which, although comparatively small, is too wide to be neglected for analytical work.

Graph or Table.—Once the correlation between needle indications and concentration values has been established for a given determination, this correlation

can be laid down in the form of a graph or in the form of a table. When operating with a graph, one has a choice of employing either ordinary coordinate paper or semi-log paper, the latter offering the advantage that straight lines rather than curves are obtained in most instances. In colorimetric routine work, it is very convenient to have the calibration in the shape of a table which indicates the concentration value for each scale reading. To prepare a table of this kind, it is necessary only to plot a graph, to draw a straight line or a curve, and to determine thereby the intermediate values which are to be entered into the table. Graph or table should be checked at least once a week.

Calibration Furnished by the Manufacturer.—Some manufacturers of photoelectric colorimeters offer calibration cards containing the complete calibration as established at the factory laboratory.

It is a sound plan for the user of the photoelectric colorimeter, even when operating with calibration cards furnished by the manufacturer, to make his own calibration tests and calibrate his instrument himself.

Accuracy of Results.—The final accuracy of the result, in terms of concentration, depends partly upon the accuracy of the chemical procedure and partly upon the precision of the instrument proper. It is to be borne in mind that a photoelectric colorimeter is not furnished with a concentration scale in the sense in which, for instance, a thermometer is provided with a temperature scale. The scale of a photoelectric colorimeter indicates transmission values, but these values serve only for translation into concentration values by means of an empirical calibration. Therefore, disregarding at this point the possible errors which may enter in calibrating and in the chemical procedure, the only limitation of accuracy is the reproducibility of the instrument readings. If a photoelectric colorimeter is in proper working condition, its reproducibility of readings should be better than one division and can, as a rule, be relied upon to be within one-half division of the 0-100 transmission scale.

The range in which a spectrophotometer should be used is between 0.2 to 0.5 optical density.

In terms of concentration, the meaning of a reading difference of one-half division depends upon the portion of the scale in which the reading is taken. At mid-scale, i.e., around 50 per cent transmission, one-half division corresponds to 1.5 per cent in concentration. At 80 per cent transmission, the corresponding value is 3 per cent and at 10 per cent transmission it is 2.5 per cent. It will be noted that the meaning of one-half division, in terms of concentration, is smaller in the center portion of the scale than on both ends. For this reason, a chemical procedure should always be planned so that the concentration range of greatest interest does not give readings near either end of the scale.

The foregoing refers to the instrument proper. The deviations attributable to the instrument being of the order of 1.5 per cent, it will be obvious that the errors which may occur in the chemical manipulations are always the larger ones. For instance, when the procedure specifies several steps involving volumetric manipulations, it will hardly be possible to keep the cumulative volumetric error from being larger than that of the instrument.

The question regarding the final accuracy of the results in terms of concentration, therefore, depends mainly upon the care exerted in the chemistry of

the calibration and the test procedure. Determinations requiring a larger number of steps of chemical manipulation will generally provide less accuracy than those comprising only a few simple steps. Also, not all the various colorimetric methods are of the same degree of conclusiveness. While some methods are completely conclusive in their results, others are inherently limited in their accuracy, due, for instance, to unavoidable interferences. In other words, each determination should be judged separately regarding the accuracy of its results. By studying the details of the chemical procedure, the user will be able to form an opinion of what accuracy can be expected.



Fig. 84.—Lumetron model 400-A, photoelectric colorimeter. (Courtesy Photovolt Corporation, N. Y.)

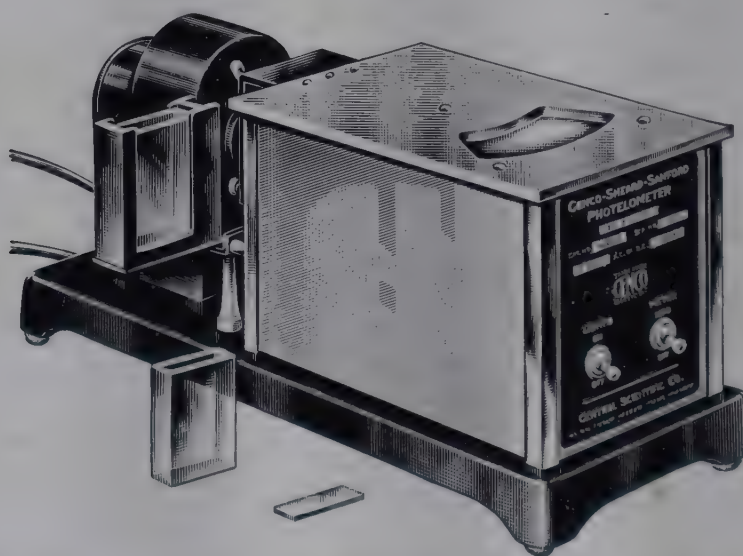


Fig. 85.—The photelometer of Sheard and Sanford. (Courtesy Central Scientific Company, Chicago, Ill.)

The Photoelectric Colorimeter

The Use of the Photelometer.—There are several excellent varieties of photoelectric cell colorimeters now on the market. Fig. 84 shows the Lumetron made by the Photovolt Corporation, New York 16, N. Y. Fig. 85 shows a view of the photelometer of Sheard and Sanford made by the Central Scientific Company of Chicago. Fig. 86 is a picture of the Leitz photoelectric colorimeter manufactured by E. Leitz, Inc., New York. Fig. 87 shows the Fisher electrophotometer, made by Fisher Scientific Company, Pittsburgh.

An electric photometer enables one to measure the absorption of light passing through a solution—even though the solution is pale or dark, and even though the differences are indiscernible to the eye.



Fig. 86.—The Leitz photoelectric colorimeter. (Courtesy E. Leitz, Inc., New York, N. Y.)



Fig. 87.—The Fisher electrophotometer. (Courtesy Fisher Scientific Co., Pittsburgh.)

Principle.¹—White light from an incandescent source can be dispersed by a prism or grating into the separate waves of all wave lengths that affect the sense of sight, and these form the visible spectrum. Both visible and invisible waves are emitted from an incandescent source. If white light is passed through a colored glass or colored solution, some of the incident light is absorbed and the rest is transmitted. The transmitted portion takes up what is called the color of the glass or the solution. “Absorption bands” are present

¹Revised from *Photometric Clinical Chemistry*, by William S. Hoffman, copyright, 1941, by William Morrow and Company, Inc., by permission.

in a solution which absorbs greater or lesser amounts of light in the narrow portions of the spectrum. These bands can be seen in the spectroscope but the actual degree of absorption can be determined only with a spectrophotometer. With this instrument, one may measure transmission percentage for a large enough number of wave lengths in the whole range of the spectrum to permit the plotting of a continuous absorption curve. Each colored solution produces a definite spectrophotometric curve. The ratio of the intensity of the light transmitted through the solution to that of the incident light is called the transmission factor. "Bouguer and later Lambert investigated the relationships between transmission factors and the corresponding thickness of homogeneous substances and found that the negative logarithm of the transmission factor is directly proportional to the thickness. Beer extended Bouguer's work to solutions of substances and found that the negative logarithm of the transmission factor of a solution of constant thickness varies as the concentration of the substance. These findings are usually expressed in the form of an equation

$$C = -\frac{1}{K} \log \frac{I}{I_0}$$

where C is the concentration of the colored substance in the solution, I_0 the intensity of the incident light, I the intensity of the transmitted light, and K a constant called the extinction coefficient, which constant is characteristic of the substance, provided the same thickness of the solution is always used. This equation is called the Bouguer-Beer law (also, but erroneously, the Lambert-Beer law)."

The photelometers of various manufacturers are made in much the same fashion. There is an optical system operated from house current by means of a constant voltage transformer, an iris diaphragm, and a convergent lens, and various types of cells and filters. Often five different filters are used; namely, blue, green, red, orange, and clear. The blue, green, and orange filters are sufficient for the tests commonly used in the clinical pathologic laboratory.

Technic of Using a Photelometer.—This technic varies with different instruments but in general the information given by the manufacturer will suffice to clarify the use of any particular make of instrument. Briefly, the needle on the microammeter should be at zero. Then with the lights off but with the meter switch on "read," place a piece of opaque cardboard in the place for the filter. The pointer should read exactly zero. If it does not, adjust it by turning the adjustment screw. An absorption cell containing distilled water is placed in the middle compartment of the cell holder, with no air bubbles and with the faces clean. An absorption cell containing the solution to be read is placed in one of the other compartments. The column of liquid should be high enough so that the meniscus is not involved in the light path. The faces of the cell should be wiped clean with a lintless, soft cloth. Place the proper filter in its place in front of the photocell. Close the iris diaphragm and turn on the light and meter switches. With the point of the fine adjustment knob turned upward, open the iris diaphragm until the pointer reads slightly more than 100. Allow several minutes to elapse before attempting the final adjustment of the pointer. Open the iris diaphragm again until the pointer reads approximately 100, and with the fine adjustment knob, set it exactly at 100 for the distilled water. Slide the cell containing the solution to be examined into the light path. The pointer moves quickly to a value less than 100. Tap the side of the case lightly before making the reading. Slide the cell with distilled water back in the light path, and see if its reading is still 100. If it is not, adjust

with the fine adjustment and read the colored solution again. Always make two readings of both the distilled water and the colored solution in quick succession in order to avoid any error due to the fatigue of the photocell.

With this instrument, accurate determinations may be made of hemoglobin, glucose, total nitrogen, nonprotein nitrogen, urea, uric acid, creatinine and creatine, cholesterol, chloride, phosphate, serum phosphatase, calcium, magnesium, sodium, etc. In other words, there is practically no limit to the usefulness of this type of instrument.

Evelyn Photoelectric Colorimeter¹

The Evelyn photoelectric colorimeter is designed to bring about, in addition to accuracy and sensitivity, (1) elimination of the use of standard solutions, (2) elimination of the subjective factor inherent in visual comparative measurements, and (3) extension of the method to deal with very pale colors, unstable and rapidly changing colors, and determinations complicated by the presence of interfering colors or turbidities.

This instrument is a single-photocell direct-reading photoelectric photometer equipped with light filters. The source of light is a selected Mazda bulb mounted in a hemispheric reflector. The photocell is of high sensitivity at the violet end of the spectrum, in which region many important measurements on yellow solutions must be made. The galvanometer is of the Rubicon multiple-reflection "spotlight" type with a sensitivity of approximately 0.008 microampere per millimeter scale division. The absorption tubes are specially selected 7 by $\frac{7}{8}$ inch round-bottomed test tubes instead of the more conventional rectangular cemented glass cells.²

Application of the Colorimeter.—The colorimeter measures solutions of single colored substances, and permits accurate measurements upon solutions of such paleness that visual comparisons are impossible. This is particularly evident with solutions which absorb energy only at the extreme ends of the visible spectrum. It is of value in the study of reaction velocities in rapidly changing systems and in the analysis of the effect on color reactions of important variables, such as time, temperature, pH, and concentration of reagents. The application of the apparatus to accurate detection of end points of titrations is important in titrations on solutions which possess inherent color.

In many instances the filters are used not to increase but to decrease the sensitivity of the apparatus to allow measurements on highly concentrated solutions in a number of procedures in which there is no shortage of chromogenic material, and the color reaction proceeds most satisfactorily at high concentrations of the colored compound.

When the color to be measured is obscured by the presence of extraneous colors present in the material on which the test is made or in the reagents used, this interfering effect may be entirely eliminated by the use of a suitable blank.

In more complicated determinations upon solutions containing an interfering color, which is developed by an unavoidable side reaction which cannot be duplicated in a blank tube, accurate measurements can still be made by the use of two filters for each determination. The same principles are employed in the

¹Made by the Rubicon Company, Ridge Avenue at Thirty-fifth St., Philadelphia 32, Pa.

²Details of operation may be found in the *Journal of Biological Chemistry* 115: 63, 1936.

analysis of a mixture of any two colored substances in solution when both components are to be measured. These measurements can be made even when the unwanted color entirely masks the presence of the color to be measured.

Extraneous and interfering turbidity may be compensated for by use of a suitable blank or by the use of two filters for each determination.

Quantitative measurements of turbidity can be accurately made on the instrument; these form the basis of a number of technics in which the material to be determined is precipitated and determined turbidimetrically.

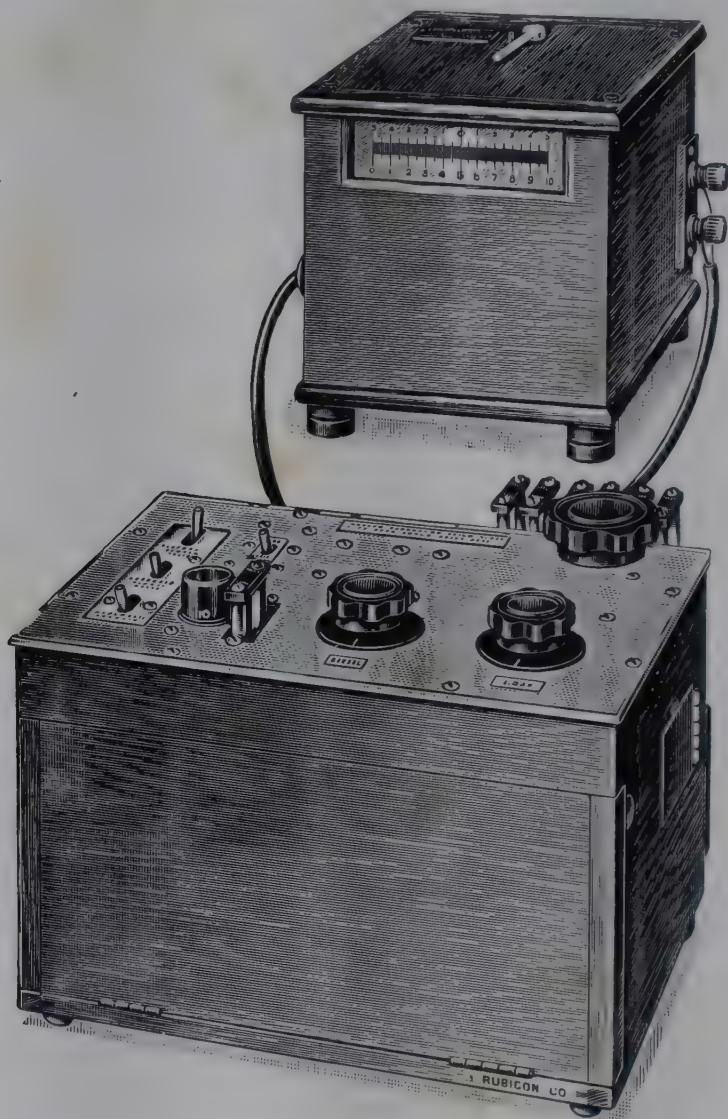


Fig. 88.—The Evelyn photoelectric colorimeter. (Courtesy the Rubicon Co., Philadelphia, Pa.)

The above applications involve the quantitative measurement of light as an index of the amount of substance present. Another important use of the apparatus is the qualitative specification of the color of solutions. Although the most rigorous method of complete specification of a color is by means of a spectrophotometric curve giving the percentage transmission at each wave length throughout the visible spectrum, nevertheless, data which are accurate enough for many purposes may be obtained with the photoelectric colorimeter by making measurements with each of a number of highly selected filters at regular intervals throughout the visible spectrum. Such measurements are valuable for the comparison of colored solutions which cannot be brought together at the same place or at the same time for visual comparison. A special application of such qualitative measurements is the determination of pH by means of indicator solutions. In this case only two filters are required for any given indicator.

Operation of the Evelyn Colorimeter¹

Select the proper filter. Insert a blank tube containing pure solvent and adjust the rheostats until an initial deflection of 100 divisions is obtained. Replace blank tube by the sample tube. Note the new deflection and obtain the corresponding value of the concentration from the proper calibration chart. After each reading the galvanometer returns to the original center setting, and the operator soon develops the habit of almost automatically checking its stability before inserting a new tube.

In calibrating the apparatus, make up carefully in triplicate a series of standards of different strengths to cover the desired range and plot the best curve possible through the resulting points. No attempt should be made to read the galvanometer more closely than to the nearest half division, since this furnishes as much accuracy as can be expected from most colorimetric procedures. Since no color standards are required from day to day, the net result of this method of calibration is that the accuracy of each day's results is maintained at a level corresponding to the greatest accuracy attainable under the most favorable conditions.

A standard should be run, if possible, every time a test is performed.

FLAME PHOTOMETRY

The use of the flame photometer is discussed on pages 414 ff.

THE SPECTROPHOTOMETER

A spectrophotometer is an instrument which produces colored light of any selected color and also measures the intensity of this monochromatic beam. The device which produces the colored light is usually called a "monochromator" and is usually calibrated to express the color of the monochromatic light it produces in terms of wave length. The device which measures the intensity of the monochromatic beam produced by the monochromator is called a "photometer." Photometric measurement is usually made both with a reference and a control sample interposed in the light beam. The difference between the two intensities is a measure of the transmittance of the sample at the wave length of the test.

Transmission.—The transmission of the tube is the ratio of the intensity of the exit beam to that of the incident beam. It is an expression of the total effect of the intercepting transparent body on the light beam. This includes both reflection losses and actual absorption by the body itself.

Transmittance.—If the transmission of distilled water is measured and then a soluble colorant is added, the intensity of the exit beam will fall from the original value to some lower value. The ratio of the intensity of this exit beam with the colorant added to that without the colorant is called the transmittance (T) of the colorant. This is an expression which depends only on the nature and amount of the added colorant independent of reflection losses and absorption losses from all other constituents of the solution.

This independence of measurements is achieved in spectrophotometry by the use of optically identical cuvettes. One is filled with a solution either free from or containing a known proportion of the constituent. This cuvette of solution is called the "reference."

Another of the identical cuvettes is filled with the solution being tested, and containing an unknown proportion of the constituent but otherwise op-

¹Evelyn, K. A.: J. Biol. Chem. 115: 63-75, 1936.

HOW A SPECTROPHOTOMETER WORKS



Ordinary light

passes through the prism and is spread into its spectrum of colors.

For visible light, a regular light bulb is used. Ultraviolet and infrared require special radiation sources. A large number of all measurements are made in the ultraviolet and infrared regions of the spectrum.

The desired color (wavelength) is selected by a knob which rotates the prism, causing the spectrum to move along the slit so that a different band of color falls on the slit opening and reaches the sample.

A slit blocks off all but a narrow band of light.

The slit width is adjustable, allowing any band width of color to be chosen. The slits can be made extremely narrow to give high "resolution" — an important performance feature of a Spectrophotometer.

The sample absorbs a portion of the light; the rest is transmitted through the sample

Some of the light rays which enter the sample displace atoms from their normal position in a molecule and make them vibrate. The wavelengths which cause this vibration are absorbed by the sample.

and strikes a phototube

Light passing through the sample strikes the phototube where it is changed into an extremely small electrical signal. The signal is then sent to an amplifier, similar to the one in an ordinary radio.

which sends a signal to the amplifier. This small signal is amplified and positions a

By greatly increasing the strength of the minute signal from the phototube, the amplifier eliminates the need for delicate parts, permitting an instrument design which is resistant to shock and vibrations.

meter which reads the amount of light passing through the sample.

The amplified signal positions the needle of an accurate meter to read the exact amount of light passing through the sample. The meter scale can be read directly in either transmittance or absorbance units.

Transmission (Absorption) Measurements. The drawing above shows how the Spectrophotometer measures the amount of a particular color (wavelength) of light absorbed or transmitted by the sample substance. By making a series of such measurements and using different wavelengths of light, the analyst can draw a curve (see Figure 2) which shows the exact location and degree of the absorptions of the sample over a wide range of wavelengths. This curve is the "absorption spectrum" . . . the unmistakable "fingerprint" of the sample.



Figure 2. The Absorption Spectrum — The Sample's Molecular "Fingerprint"

Reflection Measurements. The narrow band of color passing through the slit enters the Reflectance Attachment (see Figure 3) and is directed downward onto the opaque, colored sample. The sample absorbs some of the light. The rest of the light is reflected, strikes the phototube and is registered on the meter. The "reflectance curve" (see Figure 4) is the result of a series of such measurements made at different wavelengths. It is the only true picture of a color.

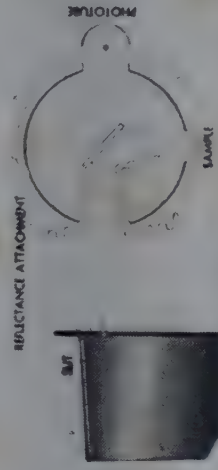


Figure 3. How Reflectance Measurements Are Made With a Spectrophotometer and Reflectance Attachment.

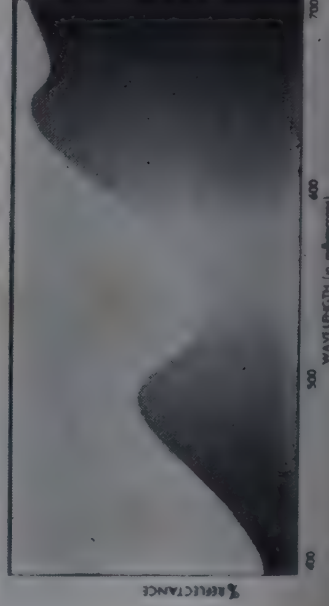


Figure 4. Reflectance Curve of a Salmon-Colored Building Tile

Flame Measurements. The sample, in liquid form, is atomized into a hot flame (see Figure 5) which acts as the light source for the Spectrophotometer. This flame excites atoms in the sample, causing them to emit radiations at various wavelengths. By measuring these emissions, the Spectrophotometer enables unknown elements to be identified and amounts of known elements to be determined. Called "flame photometry," this method is extremely rapid (as fast as 10 seconds a measurement) and to date has been applied to over 50 elements and 700 substances.

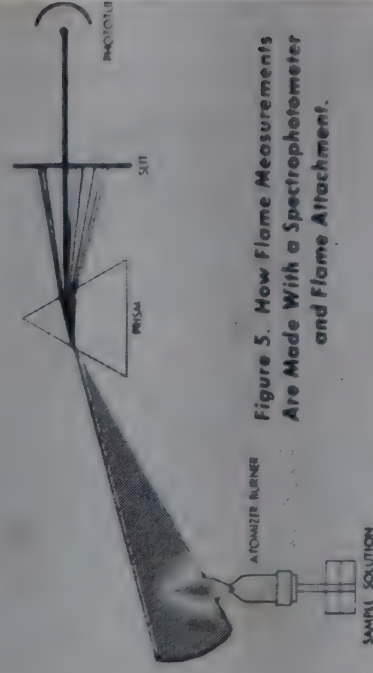


Figure 5. How Flame Measurements Are Made With a Spectrophotometer and Flame Attachment.

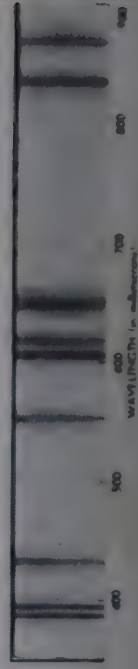


Figure 6. Emissions of Calcium When Excited in a Hot Flame

tically identical with the reference. This is called the "sample," or the "unknown." The spectrophotometer determines the difference in concentration between the reference and the sample by measuring the relative intensities of the exit beam first with the reference and then with the sample in position. . Thus in spectrophotometry, one is dealing with the transmittance of the difference in concentration of a constituent between reference and sample and not with absolute concentration.

The reference solution usually consists of a "blank" which is identical in composition with the sample except that the "unknown" is omitted. The reliability of the method depends to a great extent on the selection of the correct reference.

When a colorant is used for analysis, the amount of light which it permits to pass is always less than the amount of light which will pass through distilled water, because the colorant "absorbs" some of the light and permits the rest to pass through. The ability of a substance to interfere unequally with the passage of different colored light is called "selective absorption." Because of this selective absorption, the transmittance of a colored solution will depend on the color of the light used for measurement; the presence of a constituent will be most evident when the T (transmittance) measurement is made with light of that color most strongly absorbed by the constituent. The analysis is then made by operating at this wave length (λ).

The wave length scale of most spectrophotometers is calibrated in millimicrons ($m\mu$). The wave length of the measuring light is controlled by adjusting the wave length knob of the instrument until the required wave length appears at the index of the wave length dial.

Preparations of Curves.—All measurements should be made under similar environmental circumstances and with controlled pH or other factors which can affect color characteristics. The operator should prepare curves of interfering constituents and then select a wave length where the proposed method will be most sensitive to the concentration of the constituent and least sensitive to interference.

If the photometric measurements are made with monochromatic light having the same wave length as the characteristic absorption band of the constituent, the data almost invariably will obey the Lambert-Beer law.

The **S-T curve** (spectral-transmittance) is prepared by measuring the transmittance of a single sample at a series of wave lengths within the range of the spectrophotometer, using a suitable reference. The data obtained are then plotted on cross-section paper with T as ordinate and expressed as per cent, and wave length as abscissa and expressed as millimicrons. The principal importance of the S-T curve is to indicate that wave length at which the constituent has minimum transmittance. If the sample is too dilute or the cuvette too small, the amount of constituent intercepting the light beam may be insufficient to produce an exactly measurable response. If the sample is so concentrated or the cuvette too wide, the S-T curve may be so flattened at the region of minimum transmittance that the optimum wave length cannot be exactly ascertained.

The curve will be most indicative when the sample concentration and depth are so adjusted that the minimum T value will fall between 5 and 90

per cent. Thus the first step in the preparation of S-T curves is the selection of a suitable cuvette size and solution concentration. By preparing oneself to make these S-T curves, new spectrochemical methods can be applied to chemical analyses.

Concentration-Transmittance Calibration Graph.—

All T measurements must be made with monochromatic light.

The T measurement must be made at a wave length corresponding to a region of the constituent's S-T curves where T is constant, that is, at the middle of a region of maximum absorption or on a "flat" portion of the curve. The measurement should not be made on the "side" of an absorption band except under unusual circumstances.

The reference must be selected so that concentration equals zero when T is 100 per cent.

The T of the sample must respond only to changes in concentration.

According to the Lambert-Beer law, the concentration of a given solution is proportional to the log of its corresponding transmittance; or, in other words, the concentration-transmittance graph of this relationship will be a straight line if plotted on semi-log coordinates. Such a concentration-transmittance graph constitutes a calibration by which the concentration corresponding to any particular T value may be conveniently determined.

In addition, this straight line will always intersect the point where concentration equals zero and T 100 per cent. It is therefore possible to prepare the C-T calibration graph of an analytical method without calculations:

Determine the T of one solution of known concentration, C.

Plot this known point on semi-log coordinates.

Draw a straight line intersecting this point and the point $C = 0$, $T = 100\%$.

This line is the C-T calibration graph of the method.

The precision depends on how faithfully the Lambert-Beer law is followed. It is advisable to prepare and measure the T of several solutions of known concentration within the contemplated range of the method. The points should fall on a straight line.

The technic of almost any determination can be so developed that the data follow the Lambert-Beer law with precision, provided that measurements are made with a true spectrophotometer. The reader is referred to Consolazio, Johnson, and Marek, *Metabolic Methods*, The C. V. Mosby Company, 1951.

The Coleman Junior Spectrophotometer

The Coleman Junior spectrophotometer is pictured in Fig. 90. This is a highly sensitive instrument, and is more specific than many instruments used in colorimetric analysis. Procedures applicable to one instrument are reproducible on all others, so that a factory-calibrated standard allows instantaneous verification of calibrations. The scales are direct reading. Blank scales allow special procedures, so that new methods may be applied to this instrument without recalibration or internal adjustment. Cuvettes used are of a wide variety, including test tubes as large as 25 by 105 mm., down to capillary tubes of 0.007 c.c. capacity.

The optical system utilizes Wood's echelette diffraction grating, the system being close-coupled to a rigid optical bench. The galvanometer is installed in a shock-absorbing mounting.

Since all manufacturers supply detailed descriptions of their instruments, these will not be given here.

To Use the Coleman Junior Spectrophotometer.—The instrument should be kept on a level surface free from extreme vibrations. To minimize slight vibrational effects, mount one round sponge-rubber pad under each instrument foot. These slight vibrations might disturb exact galvanometer readings.

Keep the instrument in a position where strong light cannot fall on its panel or into the cuvette well. A 6-312 light shield may be placed over the cuvette well during every reading to prevent outside light from striking it.

Into the cuvette well, insert a cuvette adapter of the proper size to accept the type of cuvette specified in the contemplated analytical method. Standard calibrated cuvettes may be procured from the manufacturer.

Turn on the instrument by rotating the "fine" knob clockwise until the switch clicks and the λ dial is illuminated.

Verify the galvanometer "0" setting and readjust if necessary.

Adjust the λ dial to that wave length specified in the contemplated analytical method.

Wipe clean and then insert at the proper angle in the cuvette well a cuvette containing a sufficient volume of the reference solution. This volume will depend on the size of the cuvette.

Adjust the "coarse" and "fine" knobs until the galvanometer index reads that value specified in the contemplated analytical method. Usually this will be 100% T if the black transmittance scale is used, or 0 if the red density scale is utilized.

Remove the reference cuvette. Wipe clean and then insert at the proper angle a similar cuvette containing a portion of the sample solution.

Read the position of the galvanometer index on the same (red or black) galvanometer scale as was used for the initial adjustment.

Refer to tables, curves, or scale reading for the actual concentration of the sample solution, as directed in the analytical method.

Beckman Spectrophotometer

The Beckman spectrophotometer is described on page 418. See Fig. 91.

Bausch and Lomb "Spectronic 20" Spectrophotometer

The B & L "Spectronic 20" is a diffraction grating type spectrophotometer. It is a compact, direct reading instrument. It has a band width of 20 $m\mu$, and is suitable for spectrophotometry or colorimetry. It provides monochromatic light in the range from 375 $m\mu$ in the violet to 950 in the infrared. The wave length dial is graduated at intervals of 5 $m\mu$.

There are a diffraction grating monochromator with prefocused light source and fixed slits, phototubes, electronic amplification system, and built-in transformer for AC operation, a meter for direct indication of transmission and optical density, and readily interchangeable holders for test tubes or cells.

The operation of this instrument requires only three controls which adjust the wave length setting, light intensity, and dark current or zero. Results are reproducible within 0.5%.

Linear dispersion of light is provided by a grating with 15,240 lines per inch, or 600 per millimeter. The wave length scale is approximately 185 mm. long, graduated from 350 to 1,000 $m\mu$ at intervals of 5 $m\mu$ and numbered in increments of 25 $m\mu$. Red graduations above 650 $m\mu$ are to remind the user that red-sensitive phototube and filter should be inserted.

The entrance and exit slits are of fixed dimensions; the exit slit through which the monochromatic light passes to the sample is 1.5 mm. wide by 12.5 mm. high. Stray light is limited to less than 0.1% over the entire wave length range.



Fig. 90.—The Coleman Junior spectrophotometer. (Courtesy Coleman Instruments, Inc., Maywood, Ill.)

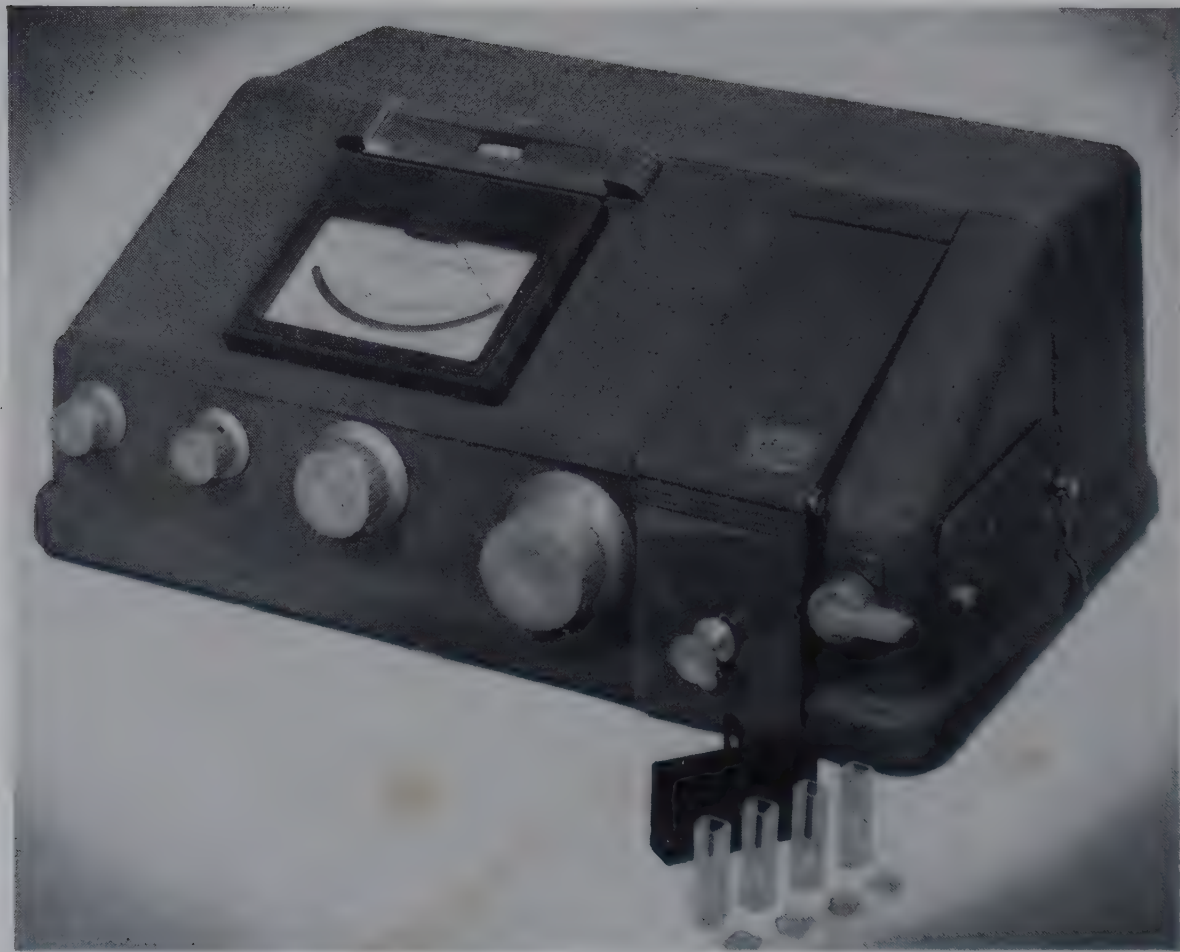


Fig. 91.—The Beckman Model B spectrophotometer. (Courtesy Beckman Instruments, Inc., South Pasadena, Calif.)

A signal from the phototube is amplified by an electronic circuit, and is indicated on a microammeter, with a scale approximately 90 mm. long, which gives direct readings in both per cent transmittance and optical density. The transmittance scale is graduated linearly from 0 to 100% in increments of 1%, numbered at intervals of 10%. The density scale is graduated logarithmically from 0 to infinite absorbance.

The amplifier circuit is of the printed, plug-in type, of simple design, and easy to remove.

The sample holder will take either a test tube approximately $\frac{1}{2}$ inch outside diameter by 4 inches long, or a square absorption cell 13 mm. wide by 13 mm. deep and 100 mm. high. Adapters are available for test tubes $\frac{3}{4}$ or 1 inch in diameter, 4 to 6 in. long, but separate lightproofing caps are required.

To facilitate dark current adjustment, a hinged shutter at the bottom of the sample chamber opens automatically when the test tube is inserted, but closes when the tube is removed.



Fig. 92.—Bausch and Lomb "Spectronic 20" spectrophotometer. (Courtesy Arthur H. Thomas Co., Philadelphia.)

Optical System.—Light from a prefocused tungsten lamp is focused by a lens onto the entrance slit. The light is converged by another lens onto the grating, which diffracts the light and reflects a uniform spectrum toward the exit slit, on the plane of which the light has been focused by the second lens. The wave length control knob rotates a metal cam which governs the angle of the grating and thereby determines the region of the spectrum which passes through the exit slit. The intensity of monochromatic beam is varied mechanically by turning the front knob on the right. When the sample is removed from the light path, the beam is automatically occluded.

The basic instrument includes a blue-sensitive phototube, operative in the range from 375 to 650 $m\mu$. For a range from 650 to 950 $m\mu$, a red-sensitive phototube and glass filter are inserted in place of the blue-sensitive tube. The red filter excludes the overlapping second-order spectrum produced by the grating.

Access to the phototube compartment is through a hinged door in the base, and conversion to the red-sensitive assembly is easily accomplished.

The Thomas Roto-Cell* provides for instantaneous interchange within the instrument of two rectangular cell chambers with 10 mm. light path, thereby facilitating preparation of data for spectral transmission or absorption curves or data.

The Roto-Cell with two cell chambers, either of which can be swiveled into the light path, can be inserted in the cell compartment in place of the single test tube holder and permits alternating the sample with the blank, or standard, by simply turning the knurled control knob. Rotation of the knob is limited by stops for positioning either cell chamber

*Made by Arthur H. Thomas Company, Philadelphia 5, Pa.

and permits blocking of the light beam to check dark current without removing the cells. The housing is so constructed as to permit attachment of a properly controlled, external cooling system to maintain the cells at optimum working temperature.

A manual of technical methods and references, directions for preparation of reagents, and calibration tables is available from the manufacturer.

ADAPTATION OF VISUAL COLORIMETRIC METHODS TO A SPECTROPHOTOMETER

The following procedure is necessary to adapt visual colorimetric methods to spectrophotometric techniques:

1. Choose the proper wave length.
2. Prepare a standard curve.
3. Change the visual colorimetric method so that the readings on the spectrophotometer are between an optical density reading of 0.2 and 0.5.

Choice of Wave Length.—The maximum point of absorption is the wave length where the greatest change in optical density occurs. Some compounds have various maxima, and it is best to choose the one which will give the straightest line when a series of diluted standards is run at that particular wave length.

To determine maxima:

Prepare the standard according to the directions of the visual colorimetric method.

Prepare a blank in the same manner by substituting distilled water in place of the standard solution.

Set the spectrophotometer at the lowest wave length and zero in the instrument with the reagent blank.

Read the standard and then reset the spectrophotometer at a wave length of 5 $m\mu$ (millimicron) more. Zero in the blank and re-read the standard.

Continue reading the standard at every 5 $m\mu$ until the end of the wave length scale.

Plot the optical density versus wave length on ordinary graph paper.

In Fig. 93, Maxima A is an optical density of 0.8, and if any unknown solution concentration is greater than the concentration of the standard, its optical density reading will be unreliable. Maxima B is an optical density of 0.3 and stronger solutions can be read in a portion of the optical density scale which is between 0.2 and 0.5. Therefore the wave length of Maxima B at 600 $m\mu$ is the one to use.

Preparation of a Standard Curve.—Prepare a series of standard solutions from 0 mg. to a few milligrams above the colorimetric standard.

React these solutions and a corresponding blank according to the colorimetric methods.

Read the standards against the reagent blank at the chosen wave length.

Plot the optical density versus concentration on ordinary graph paper.

Use the portion of the curve between an optical density reading of 0.2 and 0.5.

Final Change of Method.—Select a standard for the spectrophotometric method whose reading is around an optical density of 0.3.

Run a normal blood and check where the optical density of the solution is on the curve. If the unknown solution reads 0.1 optical density unit greater or less than the standard, change the dilution of the unknown so that the final concentration of unknown will give an optical density reading within 0.02 optical density units of the selected standard.

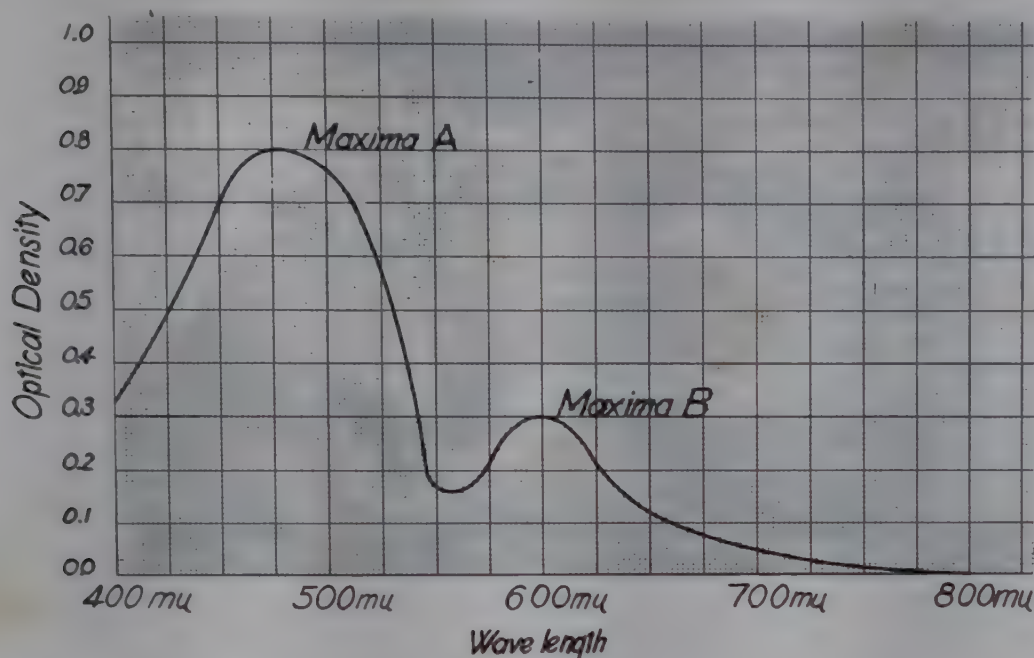


Fig. 93.—Selection of proper wave length. Refer to text, page 220.

Calculation of Results Using Spectrophotometers Instead of Visual Colorimeters.—The results may be obtained by direct reading from the standard curve, or one may set up a standard every time a test is run and calculate the results by the following formula:

$$\frac{\text{Optical density of Unknown}}{\text{Optical density of Standard}} \times \text{Concentration of Standard} \times \text{Dilution factor} = \text{Concentration of Unknown per 100 c.c.}$$

$$\text{Optical density} = 2 - \log \text{percentage transmittance.}$$

Dilution factor is that number which, when multiplied by the actual concentration of blood used in the test, equals 100.

PAPER ELECTROPHORESIS

Paper electrophoresis supplies another dimension to chromatography in general by utilizing the differences in electrical mobility of chemical substances. These differences permit separation of compounds which have like chromatographic behavior due to similarity of their partition coefficients and adsorption. See page 592 and Chapter XV, Toxicology.

Generally, in procedures for paper electrophoresis, a small sample containing the compounds to be resolved is applied to a strip of buffer-saturated paper. The paper strip is placed on a surface with its ends immersed in electrode vessels containing buffer solution. The pH of the buffer is adjusted to effect the desired migration of the compounds to be separated and at the same time to provide an electrolyte for carrying a potential. This potential is applied to electrodes located in the vessels containing buffer solution. Un-

der the influence of the voltage gradient, compounds applied to the paper will migrate at rates relative to their charges. At the end of a given time interval, the current is disconnected, the paper removed, and the separated zones located and identified by one of the conventional procedures used in paper chromatography, i.e., spraying with ninhydrin for amino acids, p-anisidine for carbohydrates, dye stain for proteins, etc. Where rigid control of operating conditions is necessary, automatic timing devices are used for disconnecting the current at the end of the desired time interval.

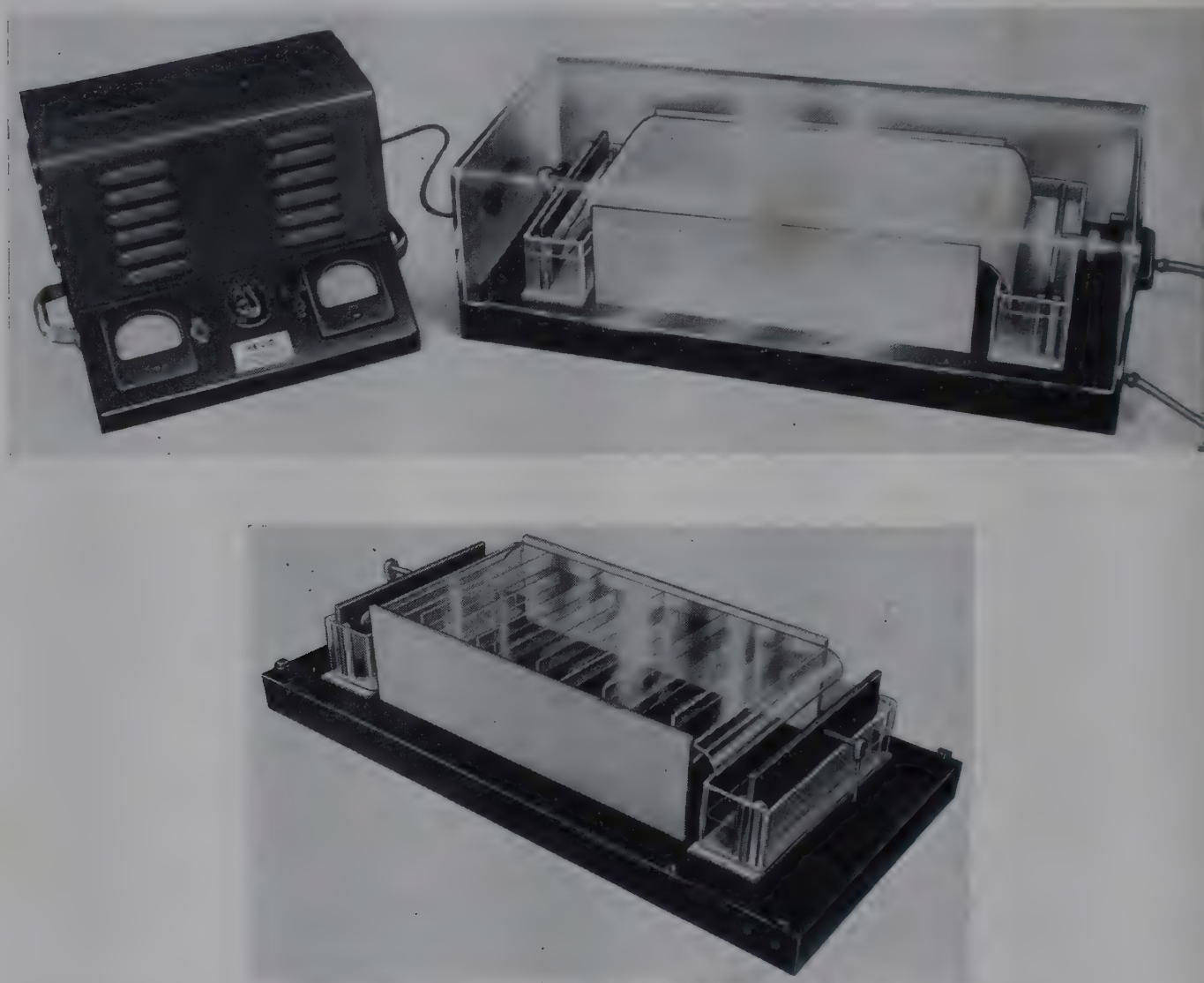


Fig. 94.—RECO Model E-800-2 electrophoresis apparatus. (Courtesy Research Equipment Corporation, Oakland, Calif.)

The **RECO** paper electrophoresis model E-800-2,* Fig. 94, consists of two units, a power supply and a Plexiglas electrophoresis unit. The latter comprises a water-cooled table (14 inches long, $8\frac{1}{4}$ inches wide, and $3\frac{1}{2}$ inches high) mounted on a black Plexiglas base; two plastic electrode vessels equipped with removable carbon electrodes and plastic diffusion barrier inserts; and a clear plastic cover. The electrode vessels, the electrodes, and the diffusion barrier plates are easily removed for cleaning. The black Plexiglas base houses the water connections for the cooling chambers, the high-voltage leads to the electrodes, and a safety switch which activates the power supply when the clear Plexiglas cover is removed.

In operation, separations can be accomplished on paper strips placed lengthwise on the cooled surface of the table with the ends dipping into the electrode vessels. In some cases it is not desirable to have the paper in contact with the table surface, and for work

*Manufactured by Research Equipment Corporation, 1135 Third St., Oakland, Calif.

of this type a one-inch high central bridge is supplied with the unit. With this device, papers can be suspended over the removable bridge in such way that contact is not made with the table surface. This application is useful for certain quantitative procedures since transfer of samples to the table surface is prevented.

Separations can also be made in solid media such as starch paste or silica gel. The solid media are confined to the table surface by sides which extend above the surface and by removable gates placed across the ends.

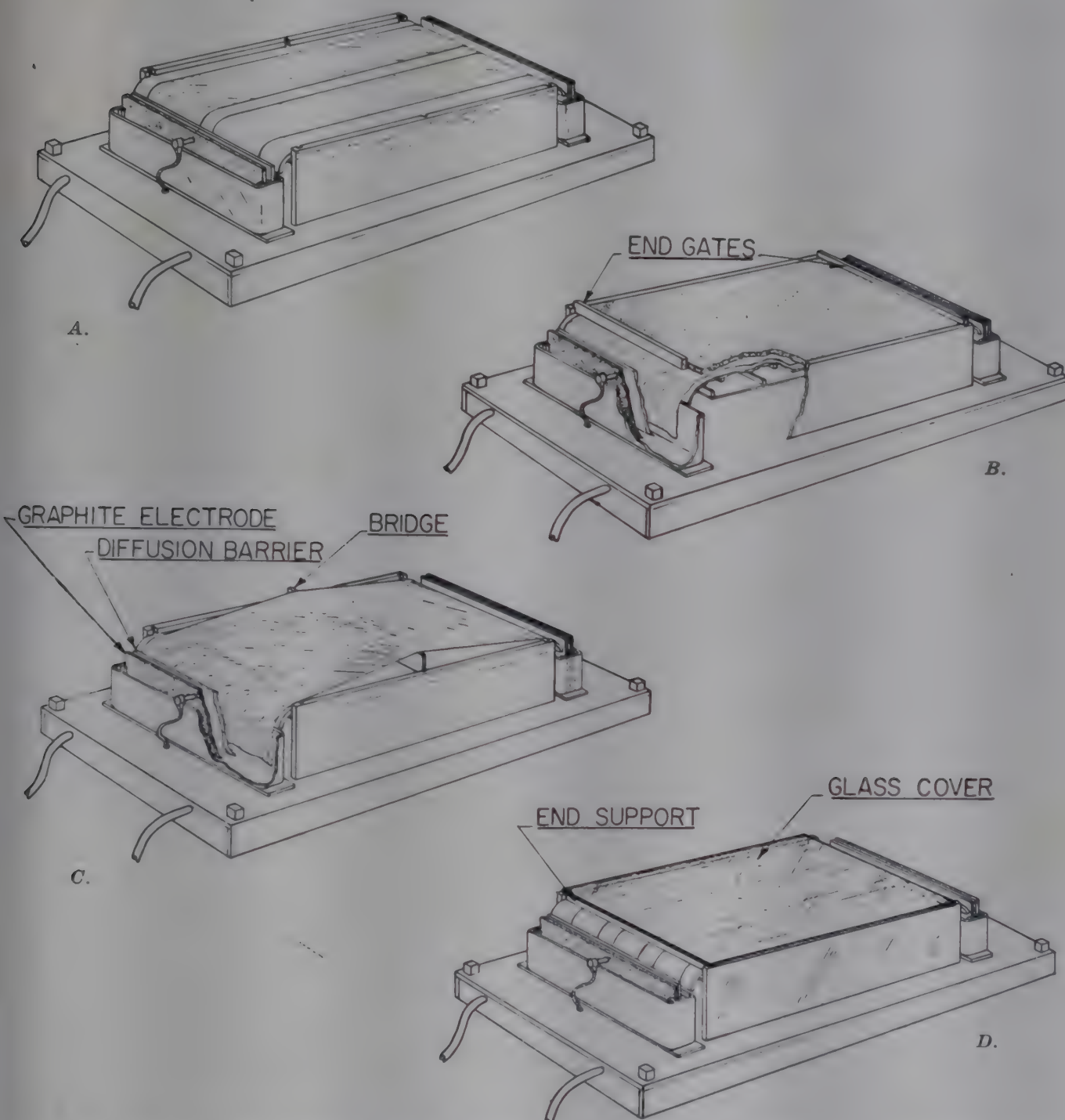


Fig. 95.—RECO Model E-800-2 paper electrophoresis apparatus: A, water-cooled electrophoresis; B, electrophoresis in solid media; C, electrophoresis with free-hanging papers; D, electrophoresis under a glass cover to minimize evaporation. (Courtesy Research Equipment Corporation, Oakland, Calif.)

The potential is applied through the electrode vessels located one at each end of the table. The equipment is designed to give a high-field strength of 53 volts per inch which results in a very rapid separation of components.

The power unit supplies up to 750 volts direct current from a full-wave rectifier, the output of which is filtered by means of a choke, capacitor network.* The output may be

*R. Consden, A. H. Gordon, and A. J. P. Martin (Biochem. J. 40: 33, 1946) point out the desirability of using a well-filtered current supply as it permits greater field strength with less heating than one which contains a much higher ripple percentage.

continuously varied from 0 to 750 volts by means of a single control and its magnitude is constantly indicated on a 3½-inch meter. The current amperage is indicated on a 3½-inch double scale meter reading 0-20 and 0-200 milliamperes. Either range may be selected by use of a two-position switch. The power supply is fused, and the primary circuits are activated by a safety switch located on the Plexiglas migration chamber base which is controlled by placement or removal of the cover. The high voltage is supplied to the electrophoresis unit by means of a 6-foot four-conductor cable from the power supply. If more than one migration chamber is to be operated simultaneously, additional outlets on the power supply unit can be furnished.

Water-Cooled Electrophoresis.—With RECO model E-800-2 one or two wide papers, or several narrow strips, may be used simultaneously, and continuously cooled during the operation by cold water circulated through the table top. Cooling the papers permits separations at high-field strengths without evaporation losses, provides more uniform buffer concentration through the length of the paper, and reduces the dangers of heat damage and denaturation of heat-labile substances during migrations (Fig. 95, *A*).

Electrophoresis in Solid Media.—Electrophoresis migrations can be carried out through a layer of solid media, such as starch paste or silica gel, spread over the surface of the table. A sheet of filter paper can be placed across the platform to serve as a wick and current conductor between the cells and the solid media, which is held in place on the surface by the slightly raised sides and the removable end gates supplied with each unit (Fig. 95, *B*).

Electrophoresis With Free-Hanging Papers.—A one-inch high removable plastic bridge (Fig. 95, *C*) which can be placed across the center of the platform to hold papers away from the surface is supplied with the apparatus. Electrophoresis on papers supported in this manner may be desirable when electrophoretic migrations can be carried out with low-field strengths and where excessive heating and evaporation losses do not occur. The method is useful for certain quantitative procedures where transfers of samples to the table surface should be avoided. See Fig. 95, *C* for relative positions of carbon electrodes, diffusion barriers, and the paper in the electrode cell.

Electrophoresis Under a Glass Cover.—The portion of the paper on the platform can be completely enclosed by a glass plate resting on the raised sides and removable end supports of the platform. This is to minimize evaporation. The cover does not touch the surface of the paper. Vaporization is confined to the shallow space directly above the paper (closed at the ends by removable plastic supports). This glass coverplate is used only in special applications, since the entire apparatus is enclosed under a plastic cover. Fig. 95, *D*.

The use of the electrophoretic apparatus in hemoglobin type determinations is discussed on page 592. Refer also to Chapter XV, Toxicology for information on iontophoresis and paper chromatography.

THE TECHNIC OF BLOOD CHEMISTRY

Distilled water is used in all blood chemical estimations. It should be made fresh several times a week and kept on hand in the laboratory, in a well-stoppered bottle, or in a bottle with a siphoning device.

Use pipettes that deliver exact quantities. Do not blow in and out of pipettes. Use Folin-Ostwald pipettes for measuring blood.

Protein-Free Filtrate, Folin

(Each c.c. filtrate contains 0.1 c.c. blood.)

The protein-free filtrate of Folin is used in all of Folin's methods of estimation of various nonprotein constituents of the blood.

Reagents.—**10% Solution of Sodium Tungstate.—**

Dissolve 10 grams sodium tungstate, Merek, Blue Label Grade, in about 50 c.c. distilled water in a 100 c.c. volumetric flask.

Dilute to 100 c.c. with distilled water.

Sodium tungstate should be neutral. In order to determine its neutrality, titrate 10 c.c. of a 10 per cent solution with phenolphthalein as indicator. Not more than 0.4 c.c. of 0.1 N hydrochloric acid should be required to neutralize it, if it is too alkaline. If it is acid, titrate to a permanent pink and add an appropriate amount of acid or alkali to the entire amount. Proteins will be incompletely precipitated if the solutions are not at the proper pH, which is around the neutralizing point. This reagent is usable for about six months.

2/3 Normal Sulphuric Acid Solution.—

Add 2 parts of N/1 sulphuric acid (see Normal solutions, page 24) to 1 part of distilled water, and mix.



Fig. 96.



Fig. 97.

Fig. 96.—Shaking the flask, preparing Folin filtrate.

Fig. 97.—End of the shaking. Note absence of foaming.

Equipment.—

- 1 250 c.c. Erlenmeyer flask.
- 1 25 c.c. volumetric pipette.
- 1 10 c.c. volumetric pipette.
- 3 5 c.c. volumetric pipettes.
- 1 4 inch funnel.
- Receptacle for collecting filtrate.
- Filter paper.

Principle.—

The total proteins of the blood are removed by precipitation with sodium tungstate and sulphuric acid, followed by filtration.

Technic.—

In a 250 c.c. Pyrex Erlenmeyer flask, place 35 c.c. distilled water.

Add 5 c.c. blood, placing the blood under the water. It is best to use Folin-Ostwald pipettes for measuring blood.

Rinse the pipette with the distilled water on top of the blood.

Mix the blood and water until hemolysis is complete.

Add 5 c.c. 10% sodium tungstate and shake. (A rubber stopper is placed in the flask between each shaking.)

Add 5 c.c. $2/3$ normal sulphuric acid, a few drops at a time, shaking after the addition of each few drops. Shake vigorously 1 minute.

The mixture should not foam. Foam means incomplete precipitation of the proteins. If the mixture is shaken vigorously until it thuds like mercury when it is shaken, the proteins will all be precipitated, and it will not be necessary to add colloidal iron. (If foaming occurs, filter through filter paper, add 3 or 4 drops of colloidal iron to the filtrate, heat until flocculence occurs, and filter. The final filtrate should not foam upon shaking.)

Let stand until a chocolate color develops, 10 minutes.

Filter through dry filter paper into a large test tube.

Collect all the filtrate, and mix well before beginning Folin's tests.

Preparation of Protein-Free Filtrate From Unlaked Blood, Folin¹

Reagents.—

$1/3$ N Sulphuric Acid—

Add 1 part of N/1 sulphuric acid to 2 parts of distilled water and mix.

Sodium Tungstate-Sodium Sulphate Solution.—

Six grams of sodium tungstate and 15 grams of anhydrous sodium sulphate are dissolved in enough distilled water to make 1 liter. The sodium sulphate should be neutral and the sodium tungstate alkaline to phenolphthalein.

Equipment.—

1 250 c.c. Erlenmeyer flask.

1 25 c.c. volumetric pipette.

1 15 c.c. volumetric pipette.

2 5 c.c. volumetric pipettes.

1 4 inch funnel.

Receptacle for collecting filtrate.

Filter paper.

Technic.—

In a 250 c.c. Erlenmeyer flask place 40 c.c. (8 volumes) of the tungstate-sulphate solution.

Add 5 c.c. (1 volume) oxalated blood.

Mix by gentle shaking and allow to stand for 5 minutes, or longer.

Add 5 c.c. (1 volume) of $1/3$ N sulphuric acid with gentle shaking.

Continue the gentle shaking for another minute.

Filter or centrifuge immediately.

The filtrate should be crystal clear.

Each c.c. of the filtrate contains 0.1 c.c. of blood.

Preparation of Protein-Free Plasma Filtrate.—

Plasma filtrate may be prepared by exactly the same procedure as is used for the unlaked blood filtrate except for the elimination of the 5-minute waiting period before the addition of the $1/3$ N sulphuric acid.

¹J. Biol. Chem. 86: 173, 1930.

Preparation of Protein-Free Filtrate, Haden's Modification of Folin-Wu Method¹

Reagents.—

N/12 Sulphuric Acid.—

Add 8.3 c.c. of N/1 sulphuric acid to 91.7 c.c. of distilled water, and mix.

10% Solution of Sodium Tungstate.—

This is the same solution that is used for the preparation of the Folin filtrate, page 225.

Equipment.—

1 250 c.c. Erlenmeyer flask.

1 25 c.c. volumetric pipette.

1 15 c.c. volumetric pipette.

2 5 c.c. volumetric pipettes.

Receptacle for collecting the filtrate.

1 4 inch funnel.

Filter paper.

Technic.—

In a 250 c.c. Erlenmeyer flask place 40 c.c. (8 volumes) of N/12 sulphuric acid.

Add 5 c.c. (1 volume) of oxalated whole blood.

Rinse the pipette with the acid blood mixture.

Allow to stand several minutes, until laking is complete.

Add 5 c.c. (1 volume) of the sodium tungstate solution.

Stopper the flask and shake thoroughly.

The coagulum should be dark brown with little or no foaming.

Filter through filter paper.

The filtrate should be crystal clear.

Each c.c. of the filtrate contains 0.1 c.c. of blood.

Protein-Free Blood Filtrate, Somogyi

Somogyi² has recommended a method of deproteinization of the blood, substituting barium hydroxide for sodium hydroxide to improve the method and expand its usefulness. This gives an excellent deproteinization method for plasma and serum. A second advantage of the use of barium hydroxide is that it introduces no salts into the blood filtrate or other tissue extracts, a desirable feature in several types of studies, as, for instance, when the extracts must be evaporated to small volumes. A third advantage of barium hydroxide is that it precipitates anticoagulants, such as fluoride and oxalate, which, if present in solution in too large quantities, may interfere with deproteinization.

Reagents.—

5 Per Cent Solution of Zinc Sulphate.—

Dissolve 5 gm. of zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) in distilled water in a 100 c.c. volumetric flask and dilute to 100 c.c. with distilled water. This solution must be titrated against the 0.3 N barium hydroxide solution as described below.

0.3 N Barium Hydroxide.—

Dilute 30 c.c. of N/1 barium hydroxide (see page 25) with hot distilled water to 100 c.c. in a 100 c.c. volumetric flask, and titrate cold against the zinc sulphate solution. Keep a drying tube containing calcium chloride on the top of the burette containing barium hydroxide to prevent formation of carbonates. Use 85.688 gm. $\text{Ba}(\text{OH})_2$ per liter for a normal solution.

¹J. Biol. Chem. 56: 469, 1923.

²Somogyi, Michael: J. Biol. Chem. 160: 1, 69-73, Sept., 1945.

The barium hydroxide must neutralize the zinc sulphate solution precisely, volume for volume, when titration is performed with phenolphthalein as an indicator. To carry out the titration, introduce 10 c.c. of zinc sulphate solution into a flask, dilute with about 100 c.c. of distilled water, then run in the barium hydroxide, a drop at a time, under continual agitation, until the phenolphthalein turns pink and the color persists for at least one minute. (Rapid titration, with the alkali running in, gives false end points.) On the basis of the titration, dilute the solution that is more concentrated to match the other.

The concentrations of these solutions are so chosen as to make their use flexible; that is, the same solutions should be suited for deproteinization of large as well as small amounts of blood, down to 0.1 c.c.

Technic of Preparing Protein-Free Blood Filtrate, Somogyi.—

For deproteinization, take the blood in a measured amount of water.

Admix the barium hydroxide solution, using 2 volumes for each volume of blood.

Add 2 volumes of zinc sulphate solution. Shake vigorously and filter. If the two reagents are correctly balanced, precipitation is perfect as manifested by the absence of foam on shaking and by the rapidity of filtration. The sugar in the blood filtrates can be determined either by the iodometric or the colorimetric method, as previously described by Somogyi.²

BLOOD SUGAR

Blood Sugar, Somogyi³

Reagents.—

Phosphate-Tartrate Reagent.—

Anhydrous disodium phosphate -----	28 gm.
N/1 sodium hydroxide -----	100 c.c.
Rochelle salt -----	40 gm.
Cupric sulphate, crystalline -----	8 gm.
Anhydrous sodium sulphate -----	180 gm.
Distilled water to make-----	1,000 c.c.

Dissolve the phosphate and tartrate in about 700 c.c. distilled water. Add the sodium hydroxide and then introduce, while stirring, 80 c.c. of a 10% copper sulphate solution. Add the sodium sulphate. When dissolved, dilute to 1,000 c.c. with distilled water and allow to stand for a day or two, during which time impurities separate out. (Or filter the solution.) Decant the clear top part of the solution and filter the remainder through a good grade of filter paper. This reagent keeps indefinitely with no sign of deterioration.

Nelson Reagent.⁴—

Dissolve 25 gm. ammonium molybdate
in 450 c.c. of distilled water.

Add 21 c.c. of concentrated sulphuric acid, c.p.

Mix.

Dissolve 3 gm. of sodium *ortho*arsenate, mono-H ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$)
in 25 c.c. distilled water.

Add this mixture to the molybdate solution and mix.

Place in an incubator at 37° C. for 24 to 48 hours. If the reagent is needed quickly, heat to 55° C. for about 25 minutes. Stir in such a way as to prevent local overheating; otherwise decomposition of the chromogen may occur. This is accompanied by the precipitation of a bright yellow compound. *Use the second method of preparation only in emergency.*

Place the reagent in a glass-stoppered brown bottle for storage.

Reagents prepared by either of the procedures probably will have considerably more of the active chromogen per c.c. than needed for 0.3 mg. of glucose. To economize on the

²Somogyi, M.: J. Biol. Chem. 160: 61, Sept., 1945.

³Somogyi, M.: J. Biol. Chem. 160: 61-68, Sept., 1945, 69-73, Sept., 1945.

⁴Nelson, N.: J. Biol. Chem. 153: 375, 1944.

reagent, one may determine in a titration against the maximum amount of glucose which it is desired to estimate how much reagent is required to give maximum color. On the basis of this, the reagent may be diluted with 1.5N sulphuric acid to allow about a 20 per cent excess concentration of the chromogenic compound over the maximum needed.

Colorimetric Method.—

The iodometric determination of reduced copper is undoubtedly superior to the colorimetric technic, but only so far as visual (Duboseq type) colorimeters are concerned, and so long as the density of the color to be measured is relatively unstable. The photoelectric measurement of color density and the development of an improved color-producing reagent by Nelson, however, have eliminated the imperfections of the colorimetric technic when it is said to be employed for microanalysis.

Mix 2 c.c. of the phosphate-tartrate reagent

and 2 c.c. of the blood filtrate in a 16 by 150 mm. test tube.

At the same time prepare sugar standards containing known amounts of glucose per c.c. (See Folin blood sugar, page 231.) Add 2 c.c. of the reagent to 2 c.c. of each of these standards in 16 by 150 mm. tubes, each tube labeled with the number and strength of the standard used.

Cover the tubes with a glass bulb (marbles are satisfactory), immerse in a boiling water bath, and heat for 10 minutes.

Cool and add 1 c.c. of Nelson's reagent.

Dilute the standards to 25 c.c. with distilled water.

Dilute the unknown to 10, 15, 20, or 25 c.c. with distilled water.

The degree of dilution depends on the density of the color. Compare in a colorimeter, setting the standard at 20. Compute by using the following formula:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{\text{Dilution of Unknown}}{\text{Dilution of Standard}} \times \text{Strength of Standard in mg. per c.c.} \times \frac{100}{\text{c.c. blood used}} = \text{mg. sugar in 100 c.c. blood.}$$

Normal: 70 to 90 mg. glucose in 100 c.c. blood (using Somogyi filtrate).

Titrimetric Method.—

Reagents.—

Phosphate-Tartrate-Iodate Reagent, Somogyi.—

To the phosphate-tartrate reagent, above, add

8 gm. potassium iodide

25 c.c. of N/1 potassium iodate (approximately 3.5%).

0.005 N Sodium Thiosulphate.—

See page 27.

Starch Indicator.—

See page 27.

2N Sulphuric Acid.—

See page 24 for normal solutions.

Technic.—

The test may be made either with 2 c.c. or 5 c.c. of 1:10 filtrate (Somogyi). For the micromethod 5 c.c. should always be used for accuracy. Reagent and filtrate are always used in equal volume.

Place in a large test tube, labeled "U" for unknown, 2 c.c. of Somogyi filtrate, page 227.

Place in a large test tube, labeled "B" for blank, 2 c.c. of distilled water.

Add 2 c.c. of reagent to both tubes. Cover the tubes.

Heat both tubes (covered) in a boiling water bath for 12 minutes.

Cool.

Add rapidly 1 c.c. of 2N sulphuric acid to both tubes, shaking the tubes while adding the acid.

Titrate with 0.005 N sodium thiosulphate using 1 to 2 drops of starch indicator. Use an accurate 10 c.c. buret with 0.05 c.c. divisions for the titration. The end point is the disappearance of the blue color.

Calculation.—

If 2 c.c. of filtrate have been used, subtract the titration “U” from titration “B” and consult Table 11 for sugar value.

If 5 c.c. of filtrate were used, consult Table 12.

Normal: 70 to 90 mg. per 100 c.c. blood.

TABLE 11.—GLUCOSE PER 100 C.C. OF BLOOD, CORRESPONDING TO TITRATION VALUES WHEN 2 C.C. OF 1:10 BLOOD FILTRATE AND 2 C.C. OF COPPER REAGENT ARE HEATED IN A WATER BATH FOR TEN MINUTES

0.005 N SODIUM THIOSULPHATE										
0.005 N	0	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.5 c.c.	0.6 c.c.	0.7 c.c.	0.8 c.c.	0.9 c.c.
THIOSULPHATE	GLUCOSE IN 100 C.C. OF BLOOD OR PLASMA									
c.c.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
0	0	11	18	25	32	40	47	54	61	68
1	75	82	89	96	103	110	117	124	131	137
2	143	149	155	162	169	175	182	189	195	202
3	209	215	221	228	234	241	248	254	261	268
4	275	281	288	295	302	309	315	322	329	336
5	342	349	356	363	370	376	383	389	396	403
6	409	416	422	429	436	443	449	456	462	469
7	476	482	489	496	503	510	516	523	529	536
8	543	549	556	563	570	577	583	590	596	603

TABLE 12.—GLUCOSE PER 100 C.C. OF BLOOD, CORRESPONDING TO TITRATION VALUES WHEN 5 C.C. OF 1:10 BLOOD FILTRATE AND 5 C.C. OF COPPER REAGENT ARE HEATED IN A WATER BATH FOR 10 MINUTES

0.005 N SODIUM THIOSULPHATE										
0.005 N	0	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.5 c.c.	0.6 c.c.	0.7 c.c.	0.8 c.c.	0.9 c.c.
THIOSULPHATE	GLUCOSE IN 100 C.C. OF BLOOD OR PLASMA									
c.c.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
0	0	4	7	10	13	16	19	22	24	27
1	30	33	36	38	41	44	47	50	52	55
2	57	60	62	65	68	70	73	76	78	81
3	84	86	88	91	94	96	99	102	104	107
4	110	112	115	118	121	124	126	129	132	134
5	137	140	142	145	148	150	153	156	158	161
6	164	166	169	172	174	177	180	182	185	188
7	190	193	196	198	201	204	206	209	212	214
8	217	220	222	225	228	231	233	236	238	241
9	244	246	249	252	254	257	260	263	266	268
10	271	274	276	279	282	285	288	290	293	296

Blood Sugar, Folin

Reagents.—

Alkaline Copper Sulphate Solution (Alkaline Copper-Tartrate Solution).—

Dissolve 40 grams anhydrous sodium carbonate, c. p., in 400 c.c. distilled water in a 1000 c.c. volumetric flask.

Add 7.5 gm. tartaric acid, c. p.

Dissolve.

Add 4.5 gm. crystalline copper sulphate, c. p., and dissolve.

Dilute to 1000 c.c. with distilled water.

Should a precipitate form after a few weeks, due to the use of impure chemicals, it may be removed by filtration through a good quality of filter paper.

Benzoic Acid, 0.25% Solution.—

Dissolve 2.5 grams of benzoic acid, c. p., in 1000 c.c. distilled water by boiling; cool and make up loss by evaporation.

Stock Solution of Glucose (1 c.c. contains 10 mg. glucose).—

Dissolve 1 gram of pure glucose, anhydrous, in 50 c.c. of 0.25% benzoic acid solution, in a 100 c.c. volumetric flask.

Dilute to 100 c.c. with 0.25% benzoic solution. The Standard Sugar Solutions Nos. I and II are made from this stock.

Standard Solution of Glucose, No. I (1 c.c. contains 0.1 mg. glucose).—

In a 100 c.c. volumetric flask, place about 50 c.c. 0.25% benzoic acid solution.

Add 1 c.c. stock solution of glucose (containing 10 mg. glucose in 1 c.c.).

Mix and dilute to 100 c.c. with benzoic acid solution.

Standard Solution of Glucose, No. II (1 c.c. contains 0.2 mg. of glucose).—

In a 100 c.c. volumetric flask, place about 50 c.c. 0.25% benzoic acid solution.

Add 2 c.c. stock solution of glucose (containing 10 mg. glucose in 1 c.c.).

Mix and dilute to 100 c.c. with benzoic acid solution.

Molybdate Phosphate Solution (Phosphomolybdic Acid Reagent).—

Place in a 1000 c.c. Pyrex beaker:

35 grams molybdic acid, c. p.

5 grams sodium tungstate (Merck, Reagent Grade).

200 c.c. of 10% sodium hydroxide solution.

200 c.c. distilled water.



Fig. 98.—Folin blood sugar tube.

Boil vigorously twenty to forty minutes to remove ammonia in molybdic acid. Cool, and transfer to a 500 c.c. volumetric flask, washing the residue in the beaker into the flask with a small amount of distilled water, until about 300 c.c. total in the flask is obtained.

Add 125 c.c. concentrated phosphoric acid (85%).

Dilute to 500 c.c. with distilled water and mix.

Equipment.—

3 Folin sugar tubes.

5 2 c.c. volumetric pipettes.

Boiling water-bath.

Principle.—

Protein-free blood filtrate is heated with an alkaline copper sulphate solution. A cuprous oxide precipitate formed by the glucose present is dissolved upon the addition of molybdate phosphate solution. This solution is compared colorimetrically with a standard glucose solution similarly prepared,

Technic.—

Use three Folin sugar tubes. Label one "U" for Unknown, one "S₁" for Standard No. I, and one "S₂" for Standard No. II.

Into tube "U" place 2 c.c. protein-free filtrate, Folin.

Into tube "S₁" place 2 c.c. standard solution of glucose, No. I, Folin (1 c.c. = 0.1 mg. glucose).

Into tube "S₂" place 2 c.c. standard solution of glucose, No. II, Folin (1 c.c. = 0.2 mg. glucose).

To all three tubes, add 2 c.c. alkaline copper sulphate solution.

Place all three tubes in a beaker of boiling water for six minutes. Remove, and place all three tubes in a glass of cold water for two or three minutes without shaking.

Add to each, without shaking,

2 c.c. molybdate phosphate solution.

Wait two minutes.

Dilute each tube to 25 c.c. with distilled water,* and mix the contents of each by inverting.

Read immediately in the colorimeter (plunger type), selecting the standard which most closely matches the color of the unknown. Standard must be darker than the color of unknown.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{"S}_1\text{" or "S}_2\text{"} = \text{the number of mg. of glucose in 100 c.c. blood.}$$

If Standard No. I is used, multiply by 100; multiply by 200 for Standard No. II

Example.—

Reading of standard, 15; reading of unknown, 10; S₁ was used.

$$\frac{15}{10} \times 100 = 150 \text{ mg. To obtain \% , divide by 1000. The \% sugar in this case is 150 divided by 1000, or 0.150\%.}$$

Normal is 80 to 120 mg. in 100 c.c. blood.

Preventing Color Fading in Folin and Wu Blood Sugar Estimations

One of the disadvantages of the Folin and Wu blood sugar test is the fact that color changes often take place and render the reading inaccurate. Lehman and Silk¹ described a method of preventing this color change. They stated that the instability remains even if precautions as to purity of chemicals are taken as described by King and Garner.²

Although the absolute loss of color tends to be greater the deeper the color, the relationship is not proportional, and it is not possible to introduce a correction. Fading is particularly inconvenient when low blood sugar values are measured, and a result of 60 mg. per 100 c.c. can give readings corresponding to 35 to 40 mg. per 100 c.c. after 20 minutes' delay. The phenomenon is largely restricted to colors of a wave length of 550 mμ and above. Thus, it is much more obvious with photoelectric color determinations where a red filter is used than with a visual colorimeter when the whole of the spectrum is measured.

The loss of color is greater the higher the temperature and depends on the amount of water added to make up the final volume.

Readings not subject to fading can be obtained by:

- (1) Cooling the Folin-Wu tubes in ice water immediately after filling to the mark, and leaving them there until measuring the color.
- (2) Using a blue or green filter which excludes the red part of the spectrum in which fading occurs.
- (3) Not diluting with water after adding the phosphomolybdate reagent.

*See suggestion on page 233.

¹Lehman and Silk: *Biochem. J.* 50: Nov.-Mar., 1951, 1952. *Proc. Biochem. Soc.*

²King and Garner: *J. Clin. Path.* 1: 30, 1947.

Additional reference: Harrison, G. A.; *Chemical Methods in Clinical Medicine*, London, 1947, J. & A. Churchill, Ltd.

All three methods have disadvantages. Working with ice water is unsuitable for routine estimations. By not registering the red part of the spectrum, one excludes the major changes in light extinction. If there is no dilution after adding the phosphomolybdate reagent, the readings become too high and the volume is too small for some types of colorimeters.

Fading on addition of water is due to lowering the phosphoric acid concentration. It does not take place if the tubes are filled to the mark with an 11.55% (w/v) solution of phosphoric acid. Dilutions to 12.5, 25, or 50 c.c. will retain their color at room temperature for more than 8 hours.

Modified Phosphomolybdate Reagent.—

Phosphomolybdate reagent of Folin and Wu	500 c.c.
Phosphoric acid, 89% (w/v)	215 c.c.
Distilled water	35 c.c.

Technic.—

Carry out the test in the usual manner.

After boiling for 6 minutes, cool for 1 minute, and add 3 c.c. of the modified phosphomolybdate reagent.

Fill to the mark with distilled water.

A final volume of 12.5 c.c. will be stable at room temperature. Even on dilution with water to 25 c.c. the fading will be below 5% in 2 hours.

Blood Sugar (Micromethod), Folin

Equipment.—

- 1 15 c.c. centrifuge tube
- 6 1 c.c. graduated pipettes
- 1 micro blood pipette, 0.2 c.c. capacity
- 2 2 c.c. volumetric pipettes
- 3 1 × 8 inch test tubes graduated at 12.5 c.c.

Reagents.—

- N/12 Sulphuric acid. (See page 24.)
- 10% sodium tungstate. (See page 225.)
- Alkaline copper sulphate. (See page 230.)
- Molybdate phosphate solution. (See page 231.)
- Standard solutions of glucose. (See page 231.)

Technic.—

In a 15 c.c. centrifuge tube place 1.6 c.c. N/12 sulphuric acid.

Add 0.2 c.c. of blood.

Rinse the pipette with the fluid.

Add 0.2 c.c. of 10% sodium tungstate.

Mix and set aside for 5 minutes.

Centrifuge for 5 minutes at 1000 revolutions per minute.

Decant the supernatant fluid or filter collecting all of the filtrate.

To a large test tube labeled "U" pipette 1 c.c. of the filtrate.

To a similar size test tube labeled "S₁," pipette 1 c.c. of the Folin standard solution of glucose No. I (1 c.c. = 0.1 mg. glucose).

To another tube labeled "S₂," pipette 1 c.c. of the Folin standard solution of glucose No. II (1 c.c. = 0.2 mg. glucose).

Add 1 c.c. of distilled water to each of the three tubes.

Add 2 c.c. of alkaline copper sulphate solution to each.

Place in a boiling water bath for 7 minutes.

Cool.

Add 2 c.c. of molybdate phosphate solution to all three tubes.

Dilute with distilled water to 12.5 c.c.

Mix by inverting the tubes and compare in a colorimeter.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times S_1 \text{ or } S_2 = \text{mg. of glucose per 100 c.c. blood. If Standard No.}$$

I is used, multiply by 100; multiply by 200 if Standard No. II is used.

Example.—

Reading of standard, 20; reading of unknown, 16; S_1 was used.

$\frac{20}{16} \times 100 = 125 \text{ mg.}$ To obtain per cent, divide this figure by 1000.

The per cent of sugar in this case is $\frac{125}{1000} = 0.125\%$.

Normal is 80 to 120 mg. in 100 c.c. of blood.

Blood Sugar, Iodometric Method of Shaffer and Hartmann***Reagents.—****Micro-Carbonate-Tartaric Acid Reagent.**

(A) Dissolve 40 grams anhydrous sodium carbonate, c.p., in 400 c.c. distilled water, warm.

(B) Then dissolve 5 grams crystalline copper sulphate, c.p., and 7.5 grams tartaric acid in 150 c.c. distilled water.

Pour B into A, stirring constantly.

(C) Dissolve 0.7134 grams potassium iodate and 10 grams potassium iodide and 18.4 grams potassium oxalate in 250 c.c. distilled water.

Rinse this into the mixture of A and B in a 1000 c.c. volumetric flask.

Cool, and dilute to 1000 c.c. with distilled water.

Starch Indicator.—

Dissolve 1 gram arrowroot starch in 20 c.c. distilled water in a mortar.

Add 200 c.c. boiling distilled water, and filter through gauze.

N/10 Sodium Thiosulphate Solution.—

See page 27.

0.005/N Thiosulphate Solution.—(1 c.c. equivalent to 0.318 mg. copper.)

Make fresh by diluting 25 c.c. N/10 thiosulphate to 500 c.c. with distilled water.

N/1 Sulphuric Acid.—(See Normal Solutions, page 24.)**Technic.—**

Place 5 c.c. of Folin filtrate (equal to 0.5 c.c. blood) in a large test tube labeled "U" for unknown.

Place 5 c.c. of distilled water in a large test tube labeled "B" for blank.

Add 5 c.c. microcarbonate-tartaric acid reagent to each.

Cover both tubes with a small inverted beaker, and place in boiling water-bath for 20 minutes.

Cool for two or three minutes under running water.

Add 1 c.c. 5/N sulphuric acid (or 5 c.c. N/1 sulphuric acid) to both tubes.

Titrate with 0.005/N thiosulphate, adding a few drops of starch indicator toward the end.

The titratable iodine is indicated as black specks when the starch is added. Titrate until the black specks disappear. This is the end point.

Wait 2 or 3 minutes to avoid mistakes due to a drifting end point.

Calculations.—

Determine the blank titration after heating the reagent with distilled water instead of blood filtrate. The blank titration of 5 c.c. of reagent should be about 20 c.c. of 0.005 N thiosulphate, and is constant.

*J. Biol. Chem. 45: 349, 1920-1921.

TABLE 13

C.C. OF 0.005/N THIOSULPHATE	TENTHS OF 1 C.C. OF 0.005/N SODIUM THIOSULPHATE									
	0	1	2	3	4	5	6	7	8	9
	MG. OF GLUCOSE IN 100 C.C. BLOOD									
0			21	23	26	29	31	34	36	39
1	41	44	46	49	51	53	56	58	61	63
2	65	68	70	72	75	77	80	82	84	86
3	89	92	94	97	99	101	103	106	108	110
4	113	115	117	119	121	124	126	128	130	132
5	135	137	139	141	143	146	148	150	152	154
6	157	159	161	163	165	168	170	172	174	176
7	179	181	183	185	187	190	192	194	196	199
8	201	203	205	207	210	212	214	216	218	221
9	223	225	227	230	232	234	237	239	241	243
10	245	248	250	252	254	256	259	261	263	265
11	267	270	272	274	276	279	281	283	285	288
12	290	292	294	296	299	301	303	305	308	310
13	312	314	316	318	321	323	326	328	330	332
14	334	337	339	341	343	345	347	350	352	354
15	356	359	361	363	365	367	370	372	374	376
16	378	381	383	386	388	390	392	394	396	398
17	400									

Subtract the titration of the test from the titration of the blank, and consult Table 13 for percentage of sugar in the blood.

Example.—

Titration of blank is 20 c.c. of 0.005 N sodium thiosulphate.

Titration of unknown is 16.5 c.c. of 0.005 N sodium thiosulphate.

$20 - 16.5 = 3.5$.

Consult Table 13.

$3.5 = 101$ mg. of glucose per 100 c.c. of blood.

Normal.—80 to 120 mg. of glucose per 100 c.c. of blood.

LaMotte Blood Sugar Outfit¹

The test is based upon the oxidation of sugar in the blood with alkaline ferricyanide and the colorimetric measurement of the ferrocyanide formed as Prussian blue. No colorimeter is used, since the resulting color of the blood test is compared with known blood sugar color standards until a color match is obtained. The test can be performed in about 20 minutes, and the use of small quantities of blood enables one to study the rise and fall of blood sugar values at close intervals. It utilizes finger blood.

Microdetermination of Blood Glucose, Method of Lauber and Mattice²

The procedure is a combination of parts of well-known and established methods. Protein removal is secured by the technic of Somogyi,³ using an alkaline zinc solution. The nature of the reducing substance in the filtrate was shown to agree closely with the fermentable sugar or "true glucose." It was estimated by the photoelectric colorimeter after development of the Folin-Wu blue color.⁴ The method is equally well suited to the visual colorimeter.

Reagents.—

The **Alkaline Copper Tartrate Solution**, **Phosphomolybdic Acid Reagent**, **Stock and Standard Solutions of Glucose** are the same as used in the method of Folin and Wu (see page 231).

¹Folin: J. Biol. Chem. 77: 421, May, 1928.

²Lauber, F. U., and Mattice, M. R.: J. Lab. & Clin. Med. 29: 113-116, 1944.

³Somogyi, M.: J. Biol. Chem. 86: 655, 1930.

⁴Folin, O., and Wu, H.: J. Biol. Chem. 41: 367, 1920.

0.25 N Sulphuric Acid Solution.—

Dilute exactly 25 c.c. of N/1 sulphuric acid to 100 c.c. with distilled water in a 100 c.c. volumetric flask.

0.25 N Sodium Hydroxide Solution.—

Dilute exactly 25 c.c. of N/1 sodium hydroxide solution to 100 c.c. with distilled water in a 100 c.c. volumetric flask. One c.c. of this solution should exactly neutralize 1 c.c. of the 0.25 N sulphuric acid.

Zinc Sulphate Reagent.—

Dissolve 6.25 gm. of zinc sulphate in about 200 c.c. of distilled water. Transfer to a liter volumetric flask.

Add 62.5 c.c. of 0.25 N sulphuric acid to the flask and mix. Dilute to 1000 c.c. with distilled water.

On titration, 25 c.c. of this zinc sulphate solution should be neutralized by 5 to 5.1 c.c. of the 0.25 N sodium hydroxide solution, using phenolphthalein as the indicator. Freshly prepared quantities of both the zinc sulphate reagent and the 0.25 N sodium hydroxide should always be standardized in this manner before use.

Paraffin Cup.—

The paraffin cup is prepared according to Abrahamson¹ by pouring liquid paraffin into a medium-sized plastic or metal bottle cap and cooling it to solidify rapidly, preferably in a refrigerator. On cooling and contraction, the surface becomes concave, forming the shallow cup. The cup has two distinct advantages. First, it serves to prevent rapid clotting of the blood; and, second, it allows several drops of blood to be pooled for pipetting without inclusion of air bubbles.

Technic.—

For determinations on capillary blood, prick the finger with a sterile puncturing needle and hold over a shallow paraffin cup so that the specimen can drip or be "milked" in this container.

To measure the blood, use a serologic pipette calibrated to contain 0.1 c.c. of blood. A convenient pipette filler can be assembled by inserting a well-fitting nut and bolt into a 3-inch length of heavy-walled rubber tubing. The opening of the tubing must fit tightly over the end of the pipette. When the tip of the pipette is dipped into the specimen, if airtight connections have been made, unscrewing the bolt will draw blood into the pipette.¹ Before discharging the measured specimen, wipe the tip of the pipette with a clean piece of gauze.

Discharge 0.1 c.c. of capillary or oxalated venous blood into 1.6 c.c. of the zinc sulphate reagent contained in a small test tube (100 by 13 mm.).

Rinse the pipette several times with the contents of this tube.

Shake the tube so that the blood is completely laked. The sample may be left at this stage, corked, at room temperature for several hours without danger of loss of sugar such as occurs with oxalated blood.

When proceeding with the determination, add 0.3 c.c. of 0.25 N sodium hydroxide. Vigorously agitate the test tube to assure uniform coagulation. After a few minutes' standing, centrifuge, and transfer 1 c.c. of the clear, colorless supernatant fluid to a graduated 10 c.c. test tube (approximately 150 by 13 mm.).

Place 1 c.c. of standard glucose solution (0.005 or 0.01 per cent according to the anticipated blood glucose value) in a similar tube, properly labeled.

Add to each tube 1 c.c. of alkaline copper tartrate reagent. Gently but thoroughly shake the tubes and heat in a boiling water bath for *exactly* eight minutes, in an upright position.

Cool in running water for one to two minutes.

Add 1 c.c. of phosphomolybdic acid. All of the cuprous oxide should react with this reagent. The tubes must not be shaken at this point. If a scum of oxide persists on the surface of the blue solution, allow an additional drop of the phosphomolybdic acid solution to fall on the film and cause its disappearance. The tubes should stand undisturbed for fifteen minutes for maximum color development.

¹Abrahamson, E. M.: Office Clinical Chemistry, New York, 1940, Oxford University Press, pp. 34, 129.

Dilute to 6.25 c.c. or some other convenient volume, and invert several times.

After four to five minutes, match in a colorimeter. It is recommended that a Klett-Summerson photoelectric instrument with a 42 filter (blue) be used. If a visual colorimeter is used, microcups and plungers should replace the standard equipment.

Special test tubes can be used for development of color. Both constricted and plain tubes one-fourth the size of the regulation Folin-Wu sugar tube have been used. The plain tube is superior to and less expensive than the constricted one.

Calculation.—

For the Klett-Summerson photoelectric colorimeter.—

$$\frac{\text{Reading of Unknown}}{\text{Reading of Standard}} \times 0.05 \times 2 \times 1,000 = \text{mg. per 100 c.c.}$$

where 0.05 = mg. glucose in 1 c.c. standard 0.005 per cent solution
 2 = factor for aliquot of filtrate analyzed
 1,000 = factor for conversion of 0.1 c.c. to 100 c.c. blood.

For the visual colorimeter (standard set at 15 mm.).—

$$\frac{15}{\text{Reading of Unknown}} \times 0.05 \times 2 \times 1,000 = \text{mg. per 100 c.c.}$$

These equations are valid when dilution of standard and unknown are the same. If the dilutions differ, an additional factor must be used to correct the preceding equations.

If the photoelectric colorimeter is used, the results can be made more precise by running a blank, wherein 1 c.c. of water is substituted for the filtrate. The reading of this blank may be subtracted from all unknown and standard readings, or the pointer of the colorimeter may be set at zero with the blank and all other solutions read with the colorimeter at this setting. The use of the blank is impractical with the visual colorimeter, since the depth of color is too small for accurate reading.

Normal.—70 to 90 mg. per 100 c.c. blood.

GLUCOSE TOLERANCE TEST

Various glucose tolerance tests have been described in textbooks of laboratory methods. Their multiplicity suggests that no ideal one has yet been devised. Gould, Altshuler, and Mellen¹ detailed their studies on the three different glucose tolerance tests now in use; namely, the 3-hour 1-dose test described by Janney and Isaacson,² the 6-hour 2-dose test of Traugott,³ and the 1-hour 2-dose test presented by Exton and Rose,⁴ further commented upon by Kelly, Bearwood, and Fowler.⁵

Exton and Rose 1-Hour 2-Dose Test

Dissolve 100 gm. of glucose in 650 c.c. of water.

Flavor with lemon and divide into two equal doses, each of which will contain 50 gm. of glucose in a 15% solution.

Prepare three containers for the blood specimens and three containers for the urine specimens.

After an overnight fast collect a sample of blood and a sample of urine.

Give the first dose of glucose to the patient.

Thirty minutes later collect the second sample of blood and give the second dose of glucose.

Thirty minutes later collect the third sample of blood and the second sample of urine.

¹Gould, Altshuler, and Mellen: *Am. J. M. Sc.* 103: 611, 1937.

²Janney, N. W., and Isaacson, V. I.: *J. A. M. A.* 70: 1131, 1918.

³Traugott, K.: *Klin. Wchnschr.* 1: 892, 1922.

⁴Exton, W. G., and Rose, A. R.: *Am. J. Clin. Path.* 4: 381, 1934.

⁵Kelly, H. T., Bearwood, J. T., Jr., and Fowler, K.: *Am. J. Clin. Path.* 5: 411, 1935.

Give the third urine container to the patient, instructing him to collect the next voiding of urine.

Estimate the amount of blood sugar in each of the blood specimens and the amount of urine sugar in each urine specimen.

According to Exton and Rose, a rise in blood sugar which does not exceed 75 mg. in the 30-minute sample is a normal response. In the 60-minute sample, the blood sugar is less than the first sample, or the same, or is not more than 5 mg. over the 30-minute sample. Normal patients should have all urine samples free from sugar. In diabetic patients, there is a rise of not less than 10 mg. of blood sugar in the 60-minute sample following the second dose of glucose. In cases of alimentary glycosuria there is a sugar-free urine after fasting, with sugar in the final urine and with blood sugar levels that follow the normal curve even when the level is higher than normal.

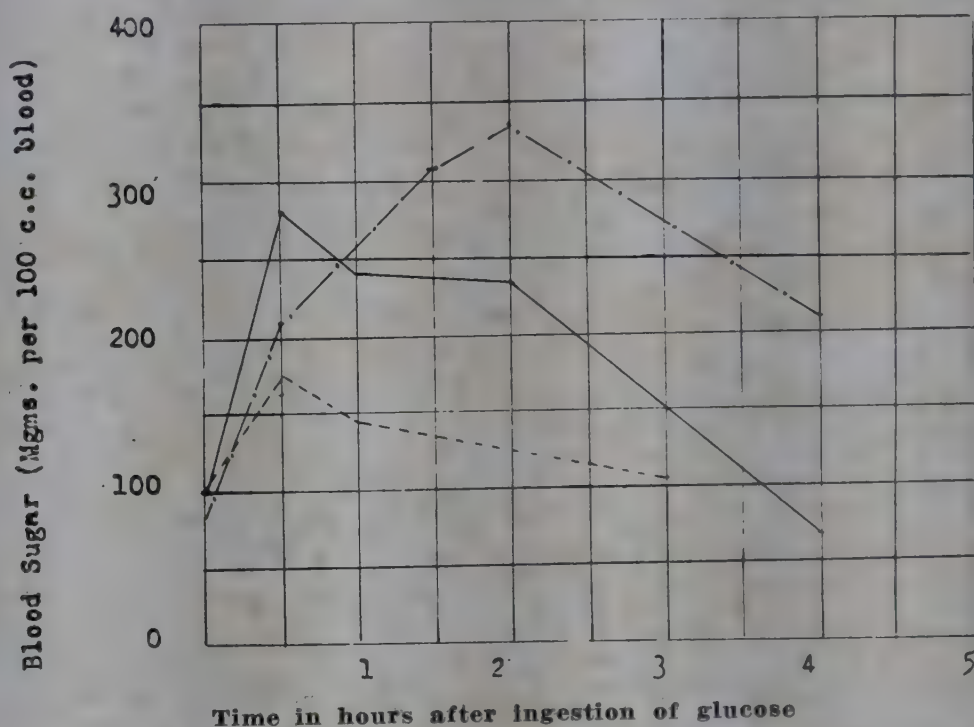


Fig. 99.—Types of curves obtained in blood sugar tolerance test. (Four-hour method.)

----- Curve of a normal individual.
 ----- Curve of a mild diabetic.
 - · - · - Curve of a severe diabetic.

(Haden: Clinical Laboratory Methods.)

Gould, Altshuler, and Mellen, as a result of their studies, comparisons, and observations, believe that the 3-hour 1-dose glucose tolerance test is not specific for the diagnosis of diabetes mellitus. They state further that by using a 2-dose test, and thus applying Allen's paradoxical law, the true glucose tolerance can be determined and the diabetic patient differentiated from the nondiabetic patient. Further, while the 6-hour 2-dose test is specific in making this differentiation, it has the disadvantages of requiring a great deal of laboratory work and of causing a good deal of discomfort to the patient. Finally, they propose new criteria for the diagnosis of diabetes mellitus by the use of the 1-hour 2-dose glucose tolerance test.

What is the 1-hour 2-dose test? This test is based upon the principle of Allen's paradoxical law, that the glucose tolerance limits in the diabetic are real while in the nondiabetic they are only apparent. In other words, in nondiabetic subjects the greater the amount of sugar given, the more is utilized; this is not the case in the diabetic.

The 1-hour 2-dose test has the advantage of being shorter and of requiring fewer venous punctures and blood determinations. Exton and Rose showed that the antecedent diet does not affect the blood sugar curves in this test. Gould, Altshuler, and Mellen have modified the technic and interpretations of Exton and Rose, by taking into consideration the weight of the patient in administering the glucose.

Instead of giving a standard amount to all patients regardless of size, retain a dosage of 1.75 Gm. of glucose per kilogram of body weight.

Divide the calculated amount into two equal parts.

Give in two separate doses.

Dissolve the glucose in water, flavored with lemon juice, 2.5 c.c. of water for each gram of glucose, making a 40% instead of a 15% solution. This obviates ingestion of a large amount of liquid.

Give half of this solution after fasting specimens of blood and urine are obtained.

At the end of 30 minutes, again obtain blood and urine samples, and immediately thereafter, give the second half of the glucose solution.

Wait 30 minutes and again collect specimens of blood and urine.

Diabetes mellitus may be correctly diagnosed if at least two of the following three conditions are encountered: (1) A fasting blood sugar which exceeds 120 mg. per cent; (2) a half-hour blood sugar which exceeds the fasting level by 50 mg. or more; (3) a 1-hour blood sugar which exceeds the half-hour level by 30 mg. or more. If a curve is obtained in which at least two of these three conditions do not exist, a diagnosis of diabetes mellitus is not justified.

The Two-Dose Glucose Tolerance Test

This test offers certain advantages over the methods described in previous pages.

The test performed is that suggested by Wayburn and Gray,¹ which is a modification of the Exton and Rose method, originated in 1931, and further modified from the method of Matthews, Magath, and Berkson of 1939.

The criteria of a normal response are that the final blood sample should be less than or only slightly higher than the peak, and that the total urinary excretion should be less than 100 mg. The present method is as follows:

Patient takes *no food* or *insulin* after evening meal, but water is allowed.

At 8:30 to 9:00 A.M. on *empty stomach*, having eaten nothing and drunk nothing except water or black coffee, patient lies down quietly for 15 to 30 minutes before beginning the test.

Fasting blood sugar and urine are now taken.

Give 325 c.c. of water containing exactly 50 gm. of glucose flavored with 2 to 3 tablespoonfuls of lemon juice.

Thirty minutes thereafter, blood and urine are taken and another 50 gm. of glucose given as above.

Thirty minutes after the second glucose, the third, and, after another sixty minutes, the fourth samples of blood and urine are taken. Qualitative and quantitative tests are made on the urine, and blood sugar determined by the Folin-Wu method.

In the case of patients having less than 158 mg. after the 1-hour test, classification is: nondiabetes; between 158 and 179 mg.: doubtful diabetes; between 180 and 260 mg.: mild diabetes; over 260 mg.: moderate to severe diabetes.

A typical nondiabetic result would be:

Fasting, 94 mg.; ½ hour, 146 mg.; 1 hour, 133 mg.; 2 hours, 114 mg.

¹Wayburn, Edgar, and Gray, Horace: Am. J. M. Sc. 204: 6, Dec., 1942.

Borderline without glycosuria:

Fasting, 105 mg.; ½ hour, 163 mg.; 1 hour, 190 mg.; 2 hours, 141 mg.

Borderline with 0.25% glucose in urine or less:

Fasting, 126 mg.; ½ hour, 201 mg.; 1 hour, 235 mg.; 2 hours, 228 mg.

Patients with 0.1 to 5 gm. sugar in the urine show in the blood:

Fasting, 120 mg.; ½ hour, 195 mg.; 1 hour, 235 mg.; 2 hours, 199 mg.

Patients with over 5 to 10 gm. show blood sugar as follows:

Fasting, 161 mg.; ½ hour, 243 mg.; 1 hour, 335 mg.; 2 hours, 334 mg.

Patients with over 10 to 15 gm. of urine sugar show in the blood:

Fasting, 199 mg.; ½ hour, 290 mg.; 1 hour, 366 mg.; 2 hours, 368 mg.

Patients with over 15 gm. of urine sugar show in the blood:

Fasting, 222 mg.; ½ hour, 323 mg.; 1 hour, 394 mg.; 2 hours, 444 mg.

The two-dose glucose tolerance test is relatively free from the influence of fairly marked changes in the composition of the preceding diet. It is superior when the antecedent diet is not known. Its superiority lies in the fact that the first dose of glucose acts as a constant balancing portion of the diet preceding the second dose.

Mosenthal and Barry,¹ in discussing the advantages of true venous blood sugar values for glucose tolerance tests over the methods using arterial blood, stated that the accepted criteria for normality in glucose tolerance tests are:

	BLOOD SUGAR MG. PER 100 C.C.
Fasting	120 or less
Ingestion 100 gm. glucose	
Subsequent highest	180 or less
Two hours after glucose	120 or less

Further, they stated that a high fasting blood sugar, as already noted (that is, over 120 mg. per 100 c.c.), is a definite sign of diminished sugar tolerance, but that a maximal blood sugar, above 180 mg. per 100 c.c., in itself is not indicative of a diminished sugar tolerance. When this is present, the blood sugar curve is called a *high curve*. When the blood sugar, two hours after the ingestion of glucose, does not return to a level of 120 mg. per 100 c.c. or less, the blood sugar curve is considered a *prolonged curve*. They called attention to the fact that every case of diabetes has a *high-prolonged blood sugar curve* but not every *high-prolonged curve* is diagnostic of diabetes. The result is that some persons are groundlessly treated for diabetes and are refused life insurance because they exhibit a high-prolonged curve, although they do not suffer from diabetes. These writers showed that high-prolonged sugar curves are often found in arthritis, hyperthyroidism, nephritis, thromboangiitis obliterans, gastrointestinal carcinoma, and epithelioma. They also occur with old age,² overweight,³ and dietary factors.⁴ In other words, in these states the sugar tolerance may indicate diabetes although it does not exist.

Mosenthal and Barry further suggested the following to avoid errors in the technic of carrying out sugar tolerance tests:

1. Avoidance of the inclusion of the nonglucose reducing substances in blood sugar determinations by resorting to methods for true blood sugar instead

¹Mosenthal, H. O., and Barry, E.: *New York J. Med.*, **46**: 2513-2518, Nov. 15, 1946.

²Deren, M. D.: *J. Lab. & Clin. Med.* **22**: 1138, 1937.

³Embleton, D.: *Proc. Roy. Soc. Med.* **31**: 1183, 1938; Short, J. J., and Johnson, H. J.: *Proc. Forty-Ninth Ann. Meet. Assoc. Life Insurance Medical Directors of America*, 1938; Spiegelman, A. R., and Mosenthal, H. O.: *Am. J. M. Sc.* **202**: 731, 1941.

⁴Conn, J. W.: *Am. J. M. Sc.* **199**: 555, 1940; Greenwald, I., Gross, J., and Samet, J.: *J. Biol. Chem.* **62**: 401, 1924; Malmros, H.: *Acta med. scandinav. Suppl.* **27**: 1, 1928; Sweeney, J. S.: *Arch. Int. Med.* **40**: 818, 1937; Tolstoi, E.: *J. Biol. Chem.* **83**: 747, 1929.

of those including nonglucose reducing substances as in the widely used Folin-Wu procedure.

2. The venous blood sugar is preferable to the arterial blood sugar in carrying out sugar tolerance tests. The arterial blood sugars are higher than the venous. The arteriovenous blood sugar difference records the assimilation of blood sugar so that the venous blood sugar values furnish the more accurate picture of sugar tolerance.

3. Consideration of the arterial blood sugar for the determination of the renal threshold to glucose, and not the venous blood sugar.

So far as methods are concerned, the Folin-Wu method includes nonglucose reducing substances. It is therefore advisable in making glucose tolerance tests to use true blood sugar determinations, of which the Somogyi⁵ and Lauber-Mattice⁵ procedures are probably the best. The Lauber-Mattice method serves for the determinations of both the capillary and venous blood sugar. It is a micromethod. The Somogyi technic is a macroprocedure and is applicable only to venous blood. It is generally believed that the nonfermentable fraction produces an error of 10 to 30 mg. per 100 c.c. of blood in this procedure.⁶ The Folin-Wu has been generally used, but Mosenthal and Barry called attention to the fact that Muhlberg⁷ in a questionnaire submitted to thirty-two of the largest insurance companies received responses from thirty, eighteen of which had home office laboratories, with the following results: The blood sugar tests used by them were: Folin-Wu, thirteen companies; Folin-Malmros, three companies; modified Folin-Wu, one company; and both Folin-Wu and Benedict, one company. Obviously, these companies do not consider the nonglucose reducing substances of major importance in evaluating blood sugars but the present writers believe that this is a mistake and that the resort to true sugar values is advisable.

The arteriovenous blood sugar difference must also be taken into account. As the arterial blood passes through the capillaries of the various tissues, some of the sugar is utilized and removed from the arterial blood. Consequently, the venous blood sugar represents arterial blood sugar less the amount of sugar metabolized by the tissues, which can be expressed as the arteriovenous blood sugar difference. In carrying out blood sugar tolerance tests, the venous blood sugar is preferable to the arterial because all the processes which contribute to the arteriovenous blood sugar difference are concerned with the better utilization of glucose and are, therefore, indicative of the degree of efficiency of the carbohydrate metabolism. Arterial blood sugar determinations are preferable only in one condition, that is, in the determination of the renal threshold to glucose and in ascertaining whether a renal glycosuria exists. It is the arterial blood which courses through the glomerular tuft and is the stimulus to filtration that takes place in that structure, while the venous blood is in no way concerned with the filtration of glucose in the production of glomerular urine.

⁵Folin, O., and Malmros, H.: *J. Biol. Chem.* 83: 115, 1929; Folin, O., and Wu, H.: *J. Biol. Chem.* 41: 367, 1920; Lauber, F. W., and Mattice, M. R.: *J. Lab. & Clin. Med.* 29: 113, 1944.

⁶Peters, J. P., and Van Slyke, D. D.: *Quantitative Clinical Chemistry*, Baltimore, 1931, Williams & Wilkins Co., vol. 1, Interpretations, Mattice, M. R.: *Clinical Procedures for Clinical Laboratories*, Philadelphia, 1936, Lea & Febiger; Sevringhaus, E. L.: *Proc. Am. Diabetes A.* 4: 119, 1944.

⁷Muhlberg, William: Personal communication.

Mosenthal and Barry insist, therefore, and very properly, that the renal threshold to glucose should be judged by true blood sugar levels obtained in arterial blood.

Significance of Blood Sugar Findings

There are three types of sugar: the "free" sugar; the loosely "combined" or colloid; and the firmly bound sugar. "Bound" sugar is transformed into "free" sugar only by hydrolysis with a dilute acid or, perhaps, by enzyme action. Free sugar of the blood is a delta glucose of the formula $C_6H_{12}O_6$. The exact identity of blood sugar has not been definitely determined, although the possibility that glucose is present in either "combined" or "bound" form has been suggested frequently. The identification of glucose has not been entirely satisfactory from a chemical standpoint from blood sugar determinations except in the case of horse blood, which was done by Hedon¹ and Hanriot.² There is evidence for the presence of "bound" sugar in blood.³ If whole blood, plasma, or serum is heated in the presence of an acid, the amount of reducing substance will increase appreciably—in human blood about 50 per cent.⁴ This has been considered by some as a polysaccharide, others call it a hydrolysis of a glycoprotein. Included in either the free sugar or loosely combined sugar fractions there are possibly some sugars other than glucose present. Dische⁵ presented evidence for the presence in human blood of two sugars besides glucose, one a ketose and the other aldose.

In 1775 Dodson was the first to recognize the presence of sugar-like substances. The celebrated experiment called "piqûre" was performed by the French physiologist, Claude Bernard, in 1848. He discovered the digestive function of pancreatic juice, and noted that by puncture of a certain part of the brain of a rabbit with a steel stylet, he was able to produce glycosuria.

Blood sugar is derived from several sources, from foods, such as carbohydrates, from protein, and from the glycerol fraction of fat. Most of the sugar derived from food is stored up as glycogen in the liver and muscles and as fat in various parts of the body. Liver glycogen is converted into sugar and discharged into the blood as the need arises. This process is under the control of adrenaline and the sympathetic nervous system. Muscle glycogen is used for muscle contraction, and does not contribute sugar to the blood.

Normal Quantities of Blood Sugar.—It is generally considered that the normal quantity of sugar is from 80 to 120 mg. per 100 c.c. of blood. The sugar content of blood varies in different parts of the circulation. Chauveau⁶ in 1856 stated that arterial blood contained more sugar than venous blood and Claude Bernard⁷ showed that the blood of the inferior vena cava just above the kidneys contained less sugar than at the level of the hepatic vein. This has been confirmed.⁸

¹Hedon: *Compt. rend. Soc. de biol.* 50: 511, 1898

²Hanriot: *Ibid.* 50: 543, 1898.

³Grevenstuck, A.: *Ergebn. d. Physiol.* 28: 1, 1929.

⁴Condorelli, A.: *Presse méd.* 35: 962, 1927.

⁵Dische, Z.: *Biochem. Ztschr.* 229: 169, 1930.

⁶Chauveau, M.: *Compt. rend. Acad. d. sc.* 42: 1008, 1856.

⁷Bernard, C.: *Leçons sur le diabète et la glycogenèse animale*, Paris, 1877.

⁸Rathery, F., Kourilsky, R., and Laurent, Y.: *Compt. rend. Acad. d. sc.* 190: 448, 1930.

Threshold Point.—The threshold point is the place where sugar flows over the kidney and appears in the urine. This threshold point is around 0.17 per cent to 0.18 per cent. It is not constant for all individuals and it varies, too, in certain diseases such as diabetes, associated with chronic kidney diseases. We have a personal record of a chronic diabetic case that had a threshold point of 0.216 per cent.

Variations Under Physiological Conditions.—In newborn babies the blood sugar is definitely lower than in later periods of life. The average for infants less than 24 hours old is 71 mg. This increases slowly until the eighth or ninth day, when the average is 82 mg. After the second week the range is practically the same as in adult life. In premature babies, there are very low concentrations.

Influence of Sex.—There is likely to be some inconstancy in the blood sugar concentration during menstruation, according to Okey and Robb.¹ Although they found no consistent cyclic variation in the fasting blood sugar values, many low as well as many high figures were observed during the menstrual period, and they advise avoiding making single blood sugar tests for diagnostic purposes during that time. During pregnancy, there may be a slight drop, followed by a slight rise during early puerperium, with a return to normal in a few weeks.

Variations Throughout the Day.—The greatest fluctuation occurs in blood sugar with respect to meals. It rises after a meal and the amount and duration of this rise is dependent upon the amount of carbohydrates taken in. Many find the highest points after breakfast. Besides the effect of meals, blood sugar reaches a very low level at midnight or before, and it remains low, during sleep, until 6 to 7 o'clock in the morning. It is advisable to take all blood sugar specimens in the morning before breakfast.

Effects of Other Factors on Blood Sugar.—Moderate exercise has no effect upon the concentration of sugar in the blood, although extreme exercise produces a marked fall. A number of glands of internal secretion interact to affect carbohydrate metabolism, principally the adrenals and the pancreas. Emotional effects are well known. Great mental shock, such as anxiety or grief may be accompanied by glycosuria. Cannon² demonstrated a case of glycosuria in students after the excitement of a football game or a difficult examination. He also found that a hyperglycemia existed in cats thrown into a great rage or fright. He explained this on the basis that these nervous stimulations affect the adrenals, causing an increased secretion of adrenaline, this, in turn, resulting in hyperglycemia and glycosuria.

Quantity of Sugar in Whole Blood, Plasma, and Corpuscles.—A number of researches have been made about this point, principally that of Tachau,³ who reported his data on the blood corpuscles and blood serum. Figures obtained by Gradwohl and Blaivas⁴ are based upon a comparison of the blood sugar content of 24 cases, using the method of Benedict and Lewis⁵

¹Okey, R., and Robb, E. I.: *J. Biol. Chem.* 65: 165, 1925.

²Cannon, W. B.: *Bodily Changes in Pain, Hunger, Fear, and Rage*, New York, 1915, D. Appleton Co.

³Tachau, H.: *Ztschr. f. klin. Med.* 79: 421, 1914.

⁴Gradwohl, R. B. H., and Blaivas, A. J.: *J. Lab. & Clin. Med.* 2: No. 6, 1917.

⁵Benedict and Lewis: *J. Biol. Chem.* 20: 61, 1915.

modified by Myers and Bailey.¹ The blood was diluted one to five with distilled water and precipitated with picric acid, using the Benedict technic.

Their figures showed that the quantity of sugar in the whole blood, in the plasma, and cells is nearly the same in all cases.

TOTAL NONPROTEIN NITROGEN

Total Nonprotein Nitrogen, Folin and Wu

Reagents.—

Standard Ammonium Sulphate Solution.—(3 c.c. contain 0.3 mg. nitrogen.)

Dissolve 0.4716 gram dried ammonium sulphate of the highest purity in about 800 c.c. distilled water in a liter volumetric flask.

Dilute to 1000 c.c. with distilled water.

Digestion Mixture.—

Mix 100 c.c. concentrated sulphuric acid, c. p.

with 300 c.c. of phosphoric acid, syrupy (85%).

Let stand at least one week.

Then to 100 c.c. of the above mixture

add 10 c.c. of 6% copper sulphate solution

and 100 c.c. distilled water.

Stock Nessler's Solution (Folin).—

Place 150 grams of potassium iodide

and 110 grams of iodine, resublimed, in a 500 c.c. Florence flask.

Add 100 c.c. distilled water

and an excess, 140 to 150 grams, of metallic mercury, redistilled.

Shake the flask vigorously and continuously for seven to fifteen minutes, or until the iodine has nearly disappeared.

The solution becomes quite hot.

When the red iodine solution has begun to pale visibly, though still red, cool in running water and continue the shaking until the reddish color of the iodine has been replaced by the greenish color of the double iodide. This whole operation usually does not take more than 15 minutes. Separate the solution from the surplus mercury by decantation and washings with successive liberal quantities of distilled water. Dilute the solution and washings to a volume of 2 liters. If the cooling is begun in time, the resulting reagent is clear enough for immediate dilution with 10% alkali and water, as follows:

Diluted Nessler's Solution (for use), or Nessler's Reagent, Folin.—

To 150 c.c. of stock Nessler's solution (above)

add 700 c.c. of 10% sodium hydroxide solution

and 150 c.c. of distilled water.

Note. Folin made the following suggestion about turbidity resulting from nesslerization. "Many seem to have trouble in obtaining perfectly clear solutions when nesslerizing the digestion mixtures obtained with blood filtrates. The cause is lack of suitable alkalinity in the Nessler's solution.

"The following data will help to overcome the difficulty: 20 c.c. of N/1 hydrochloric acid may be titrated with the Nessler solution, and if the solution is substantially correct, a good end point will be obtained at 11 or 11.5 c.c. with phenolphthalein as indicator. If an end point is obtained much below 11 c.c., as at 9.5 c.c., the Nessler solution is too alkaline, and turbidity is likely to occur.

"Turbidity due to excess of alkalinity may likewise be produced because the sulphuric-phosphoric acid mixture is too weak. If 5 c.c. of diluted acid (1 to 1) are further

¹Myers and Bailey: J. Biol. Chem. 24: 147, 1916.

diluted 10 times (to 50), 10 c.c. of the solution so obtained, when titrated with the Nessler solution and phenolphthalein as indicator, should give a fairly good end point at 9 c.c. to 9.3 c.c." Folin: Laboratory Manual of Biological Chemistry, 1925.

Gum Ghatti Solution.—

Fill a 500 c.c. cylinder with distilled water. Suspend at the top, just below the surface, in a wire basket of galvanized iron, 10 gm. of the gum ghatti. Leave to dissolve overnight but not for 24 hours. Remove the wire basket with remaining undissolved material. The clear solution is transferred to a bottle, and, as a preservative, 0.4 to 0.5 gm. of benzoic acid dissolved in 5 c.c. of alcohol, is added.

Equipment.—

- 1 Pyrex test tube (1 × 8 inches).
- 1 5 c.c. volumetric pipette.
- 1 1 c.c. graduated pipette.
- 1 100 c.c. volumetric flask or graduated cylinder.
- 1 2 c.c. volumetric pipette.
- 1 microburner and ring stand with clamp.
- 1 15 c.c. volumetric pipette.
- 1 3 c.c. volumetric pipette.
- 1 25 c.c. volumetric pipette.
- 1 10 c.c. volumetric pipette.

Principle.—

The nitrogen content of the protein-free filtrate is determined colorimetrically by digestion with sulphuric-phosphoric acid digestion mixture. The ammonia formed is determined, after nesslerization, by comparison with a similarly prepared standard nitrogen solution.

Technic.—

Use the Folin protein-free filtrate, page 224 (1 c.c. contains 0.1 c.c. blood).

In a Pyrex tube (75 c.c. capacity, 8 × 1 inch) place 5 c.c. Folin filtrate.

Add 1 c.c. digestion mixture.

Evaporate with a microburner until the solution turns brown, then clear again. To carry out this step correctly, clamp the Pyrex tube in a buret stand, and boil the mixture until it turns brown. Do not let it boil over. To prevent the boiling solution from popping, numerous glass beads may be placed in the tube, or air may be forced into it until the total quantity is very low. When the mixture turns brown, cover with a clean watch glass, and continue the evaporation process with a slow steady flame until fumes of sulphuric acid are seen. When no more of these white fumes are generated, the remainder of the fluid in the tube will be clear. Immediately remove the flame. If too much heat is employed, or the evaporation carried too far, the material will crystallize, and the procedure will have to be repeated.

Add to the evaporated liquid 35 c.c. distilled water.

Add 1 or 2 drops of gum ghatti solution.

In a 100 c.c. volumetric flask, marked "S" for standard,

place 3 c.c. standard ammonium sulphate solution, Folin (1 c.c. = 0.1 mg. N).

Add 2 c.c. digestion mixture

and 50 c.c. distilled water.

Add 2 to 4 drops of gum ghatti solution.

To the unknown in the Pyrex tube, add 15 c.c. Nessler's reagent (Dilute Nessler, Folin).

To the standard in the volumetric flask, add 30 c.c. Nessler's reagent (Dilute Nessler, Folin).

Dilute the standard to 100 c.c. with distilled water.

Mix the contents of each container by inverting several times, and read in the colorimeter.

For explanation of cloudy unknowns, see page 244.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 30 = \text{the mg. of nonprotein nitrogen in 100 c.c. blood.}$$

Example.—

Reading of standard is 15; reading of unknown is 12; results are:

$$\frac{15}{12} \times 30 = 37.5 \text{ mg. of nonprotein nitrogen in 100 c.c. blood.}$$

Normal is 25 to 35 mg. of nonprotein nitrogen in 100 c.c. blood.

In normal human unlaked blood, the nonprotein nitrogen is approximately 10 mg. lower than in laked blood, the average being about 20 mg. per 100 c.c. of blood (see page 226).

Micromethod for the Determination of Nonprotein Nitrogen, Folin

Reagents.—**Sulphate-Tungstate Solution.—**

Into a 500 c.c. volumetric flask place 10 gm. of anhydrous sodium sulphate, c.p.
Add 15 c.c. of 10% sodium tungstate solution.
Add 250 c.c. of distilled water.
Shake until the sulphate has dissolved.
Dilute to 500 c.c. and mix.

Sulphuric Acid Solution.—

Dilute 12 c.c. of 2/3 N sulphuric acid to 100 c.c. and mix.

Mixture of Sulphuric Acid, Phosphoric Acid, and Copper Sulphate.—

In a 250 c.c. volumetric flask place about 50 c.c. of distilled water.
Add 15 c.c. of 85% phosphoric acid.
Add 10 c.c. of concentrated sulphuric acid,
and 5 c.c. of 5% aqueous copper sulphate solution. (5 gm. in 100 c.c.)
Dilute to 250 c.c. with distilled water.
This solution should be kept in a glass-stoppered bottle.

Standard Ammonium Sulphate Solution.—

This solution is made by diluting 10 c.c. of the standard solution (page 244) to 100 c.c. with distilled water. Each c.c. will contain 0.01 mg. of nitrogen.

Nessler Reagent.—(Page 244.)

Gum Ghatti Solution.—(Page 245.)

Equipment.—

- 1 15 c.c. conical centrifuge tube.
- 1 special microblood pipette.
- 2 Pyrex test tubes (1 × 8) graduated at 25 c.c.
- 4 2 c.c. volumetric pipettes.
- 2 1 c.c. pipettes graduated in 0.1 c.c.
- Microburner, ring stand, and test tube clamp.

Technic.—

In a clean, dry 15 c.c. conical centrifuge tube, place 4 c.c. of the sulphate-tungstate solution.

With the special microblood pipette add 0.2 c.c. of patient's blood. Rinse with the solution in the tube.

Stir and allow to stand for 15 minutes or longer.

Add 1 c.c. of the dilute sulphuric acid solution.

Stir carefully but thoroughly, and centrifuge at fairly high speed for 5 minutes.

Decant clear supernatant fluid.

Place 4 c.c. in a Pyrex test tube graduated at 25 c.c.

Add 1 c.c. of the sulphuric acid-phosphoric acid mixture.

Carry out digestion as for the Folin-Wu method. (Page 245.)

When digestion has been completed, allow the material to cool and add 2 drops of gum ghatti solution.

Dilute to about 15 c.c. with distilled water.

Place in another graduated test tube marked "S" for the standard 4 c.c. of the standard ammonium sulphate solution (containing 0.04 mg. N).

Add 1 c.c. of the sulphuric acid-phosphoric acid mixture.

Add 2 drops of gum ghatti solution.

Dilute to about 15 c.c. with distilled water.

Nesslerize both the unknown and the standard tubes with 4 c.c. of the Nessler reagent. This should be done as nearly simultaneously as possible.

Dilute to the mark, mix, and compare colorimetrically.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 25 = \text{mg. nonprotein nitrogen in 100 c.c. of blood.}$$

Example.—

$$\text{Reading of Standard} = 20; \text{Reading of Unknown} = 30.$$

$$\frac{20}{30} \times 25 = 16.667 \text{ mg. nonprotein nitrogen in 100 c.c. blood.}$$

Normal is 25 to 35 mg. of nonprotein nitrogen in 100 c.c. blood.

UREA NITROGEN

Urea Nitrogen, Folin and Wu

Reagents.—

Urease Solution.—

By adding urease to distilled water, the urease is inactivated by the slight traces of heavy metals in the water. The best method is to add urease powder directly to the blood and then add the water. However, since the original method calls for a urease solution, the author has retained the original technic.

Add 5 grams of either Squibb's or Arlington's urease powder to 100 c.c. distilled water. The urease purchased from these firms contains the phosphate solution. Keep refrigerated.

If the tablets of Hynson, Wescott, and Dunning are used, break up 2 tablets in 1 c.c. distilled water to obtain a 5% solution.

Pyrophosphate Solution.—(This solution is not used if the urease from the above-named firms is used.)

Dissolve 14 grams of sodium pyrophosphate, U. S. P., and 2 grams of glacial phosphoric acid in enough distilled water to make 100 c.c.

10% Sodium Hydroxide Solution.—

Standard Ammonium Sulphate Solution (1 c.c. contains 0.1 mg. nitrogen).

This is the same as described under total nonprotein nitrogen, page 244.

Nessler's Stock Solution and Reagent for this test are the same as described under total nonprotein nitrogen, Folin, page 244.

Equipment.—

- 2 100 c.c. lipless cylinders.
- 1 medium-sized test tube.
- 1 5 c.c. volumetric pipette.
- 4 1 c.c. pipettes graduated in 0.1 c.c.
- 2 2 c.c. volumetric pipettes.
- 1 3 c.c. volumetric pipette.
- 1 10 c.c. volumetric pipette.
- 1 20 c.c. volumetric pipette.
- 1 set of aeration equipment.

Technic.—**Description of Chemistry and Aeration.—**

The enzyme **urease** converts urea into ammonium carbonate. The ammonia is then liberated by aeration (in the presence of an alkali in excess) and is carried over into a hydrochloric acid solution, where it is converted to ammonium chloride. Ammonium chloride can be determined colorimetrically by adding Nessler's reagent, and comparing the color produced with the color of a standard containing a known amount of nitrogen.

Two cylinders are required for each specimen of blood. In addition, a cylinder containing a solution (10 or 20%) of sulphuric acid is used as a wash bottle to remove the nitrogen (from the ammonia) from the air drawn through the cylinders in the aeration process.

If more than one specimen of blood is aerated at the same time, there are two cylinders for each specimen, and one wash bottle at the end of the apparatus. The cylinders are lined up in series, two for each specimen, the cylinder containing the hydrochloric acid being nearer the pump, and the cylinder containing the blood being nearer the sulphuric acid wash bottle at the end of the line.

The cylinder containing the hydrochloric acid must be graduated. All cylinders must be lipless.

A two-hole rubber stopper is placed in each cylinder.

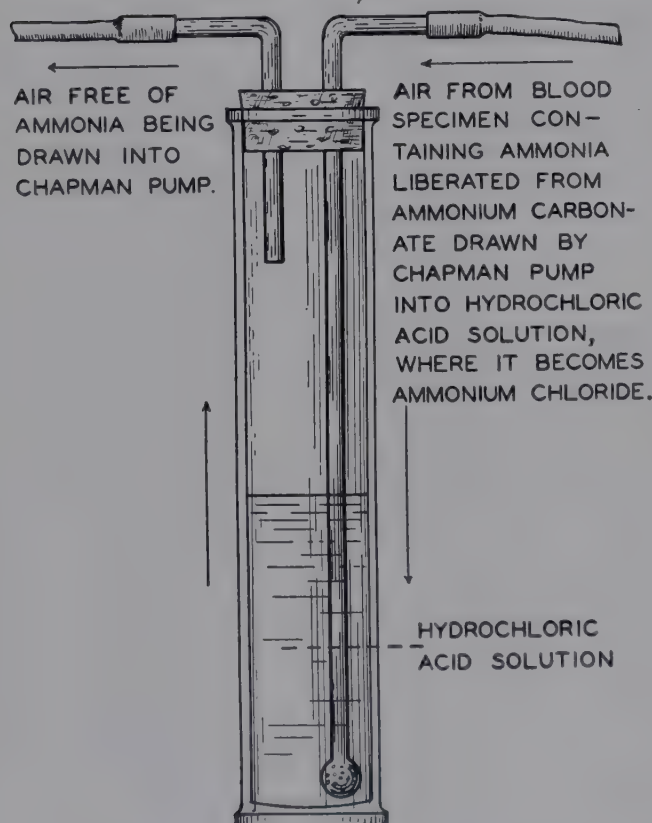


Fig. 100.

Fig. 100.—Cylinder No. 1 containing the converted urea in the form of ammonium chloride.

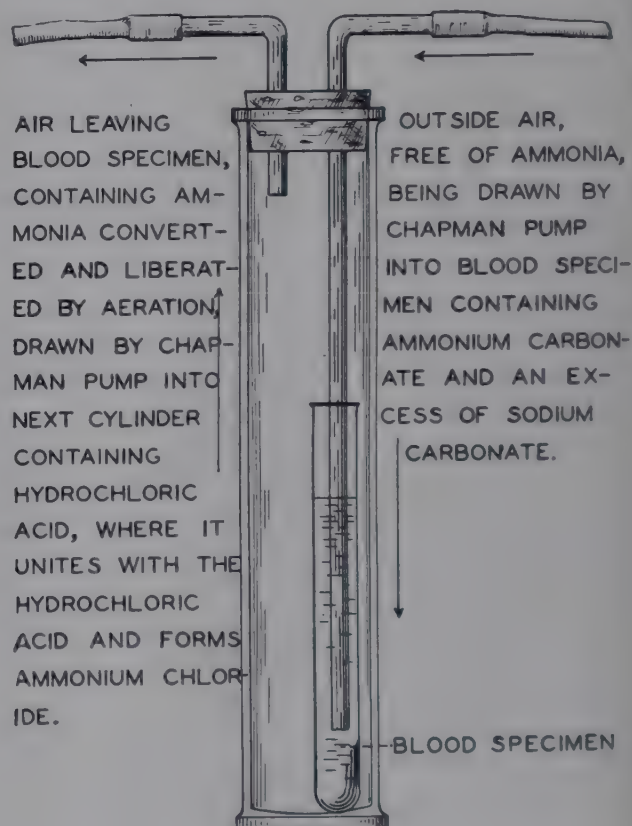


Fig. 101.

Fig. 101.—Cylinder No. 2 containing the blood specimen.

Glass Connections for Cylinder No. 1 (containing the hydrochloric acid). A piece of glass tubing, exactly fitting the hole of the rubber stopper, is bent at right angles, and placed through the hole of the stopper so that the end just extends into the cylinder. Another similar piece of glass tubing is placed in the other hole, and to this bent arm is attached, by means of a piece of rubber tubing, a glass tubing with a perforated bulb at one end. The bulb must extend into the hydrochloric acid, and must have about 6 or 8 perforations. The tubing is called the "delivery tube." The arm not connected with the delivery tube is the one which is attached to the suction.

Glass Connections for Cylinder No. 2 (containing the blood). A piece of glass tubing bent at right angles, and fitting the hole of the rubber stopper, is placed in the hole

of the rubber stopper so that it just extends into the cylinder. This arm is closest to the suction. Another glass tubing is bent at right angles and placed in the other hole, but attached to it is a straight piece of glass tubing, long enough to reach to the bottom of the test tube of blood which is to be placed in this cylinder.

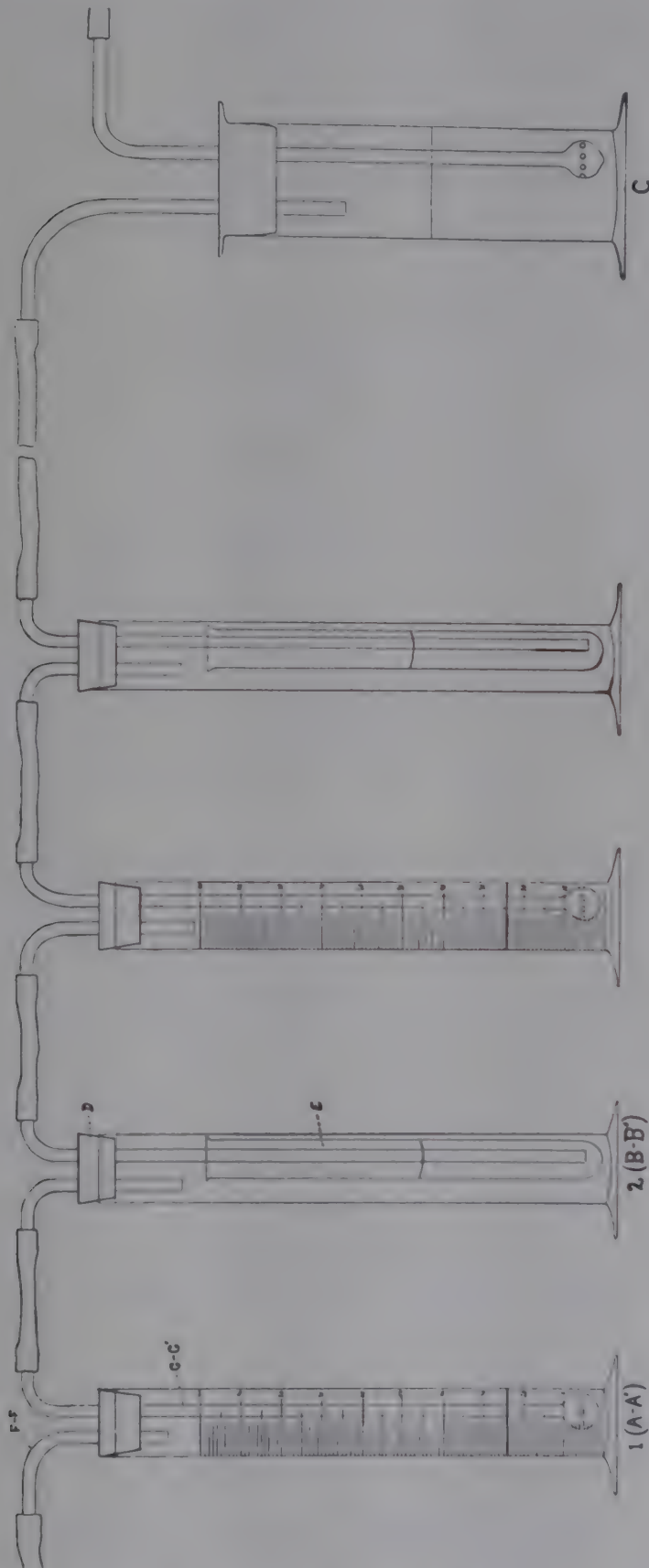


Fig. 102.—Urea apparatus set up and connected to the suction. (Gradwohl and Blaivas.)

Glass Connections for the Acid Wash Bottle may be a cylinder fitted with a two-hole rubber stopper, and two pieces of glass tubing bent at right angles. The arm closest the suction is short. The other arm extends into the sulphuric acid, almost to the bottom of the cylinder.

When attaching one blood specimen to the suction, the hydrochloric acid solution is closest, then the blood specimen, then the wash bottle. If two bloods are being examined,

the hydrochloric acid of specimen 1 is attached to the suction, then blood specimen 1; next hydrochloric acid for blood specimen 2, then blood specimen 2; last, the sulphuric acid wash bottle.

The cylinders are connected to each other and to the pump by means of rubber tubing. The short arm of each cylinder is in the direction of the pump; the long arms, those which extend into the cylinders or blood, are nearer to the wash bottle. Not more than 3 specimens should be run at once.

Technic.—

Into a test tube that will readily slip into a 100 c.c. lipless cylinder, place 5 c.c. Folin's protein-free filtrate (containing 0.5 c.c. blood).

Add 0.5 to 1.0 c.c. 5% solution.*

(If urease other than that of Squibb or Arlington Co., or the tablets of Hynson, Wescott, and Dunning are used, add 2 drops of pyrophosphate solution.)

Let stand at room temperature for fifteen to twenty minutes, or place in a water bath at 50° to 55° C. for five minutes to convert the urea into ammonium carbonate.

Add 1 to 2 c.c. of 10% sodium hydroxide solution and mix.

Add 1 c.c. antifoaming mixture, or Mallinckrodt's fusel oil.

Place immediately in cylinder 2 for aeration, and stopper with the rubber stopper.

In cylinder No. 1, place 15 to 20 c.c. distilled water and 1 c.c. N/10 hydrochloric acid.

Stopper. Be sure the glass delivery tubes of both cylinders extend into the fluid.

Connect to the suction pump for aeration, connecting the sulphuric acid wash bottle at the end. Be sure apparatus has no air leaks. Begin suction slowly at first, gradually increasing it, until it reaches the maximum.

Aerate for 30 to 45 minutes, or until all the ammonia (liberated from the ammonium carbonate) has been removed from the blood, and is in the hydrochloric acid as ammonium chloride.

Remove from the suction.

The cylinder containing the urea, now in the form of ammonium chloride, is the one which originally contained the hydrochloric acid solution, and is used in the final determination.

Place in a 100 c.c. volumetric flask, marked "S" for standard, 3 c.c. standard ammonium sulphate solution, Folin (1 c.c. = 0.1 mg. N).

Add 70 c.c. distilled water.

To the unknown in cylinder No. 1 add 2.5 c.c. Nessler's reagent (dilute Nessler's solution, Folin).

To the standard, add 10 c.c. Nessler's reagent (dilute Nessler's Folin).

Dilute the unknown to 25 c.c. with distilled water.

Dilute the standard to 100 c.c. with distilled water.

Mix the contents of both containers by inverting several times, and read in a colorimeter.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 15 = \text{the mg. of urea nitrogen in 100 c.c. blood.}$$

Example.—

Reading of standard is 15; reading of unknown is 12. Urea nitrogen is

$$\frac{15}{12} \times 15 = 18.7 \text{ mg.}$$

Normal is 10 to 15 mg. urea nitrogen in 100 c.c. blood.

These figures must not be confused with urea. To find the amount of urea, multiply the urea nitrogen figures by 2.14. Normal urea: 25 to 30 mg. per 100 c.c. of blood.

*See note under Urease Solution, page 247.

Urea Nitrogen, Gentzkow and Masen¹ (Method of Choice)

Reagents.—

10% Sodium Tungstate Solution.—(See page 225.)

0.66 N Sulphuric Acid Solution.—(See page 225—2/3 N Sulphuric Acid.)

Stock Standard Nitrogen Solution.—

Dissolve 7.074 gm. ammonium sulphate, reagent grade, in N/10 sulphuric acid in a liter volumetric flask, and dilute to 1,000 c.c. with N/10 sulphuric acid.

1 c.c. contains 1.5 mg. N.

Working Standard Nitrogen Solution.—

Dilute 5 c.c. of the stock nitrogen solution to 500 c.c. with N/100 sulphuric acid in a 500 c.c. volumetric flask.

1 c.c. = 0.015 mg. nitrogen.

1% Potassium Gluconate.*—

Dissolve 1 gm. potassium gluconate, c.p., in enough distilled water to make 100 c.c. This solution must be made weekly and stored in a refrigerator.

2.5% Potassium Persulphate.*—

Dissolve 2.5 gm. potassium persulphate, c.p., *nitrogen-free*, in enough distilled water to make 100 c.c. solution. This solution must be prepared weekly and stored in a refrigerator. Take from the refrigerator long enough only to remove whatever is required in the test because the solution decomposes rapidly at higher temperatures.

Urease, Squibb, Double Strength.—

Dilute Nessler's Solution, Folin, Modified.—

Place 500 c.c. of 10% sodium hydroxide in a liter volumetric flask.

Add 150 c.c. stock Nessler's solution, Folin (page 244).

Dilute to 1,000 c.c. with distilled water. Allow to stand for three days to permit sedimentation of the precipitate.

Nessler's Mixture.—

Note: *This solution must be used within 15 minutes of its preparation.*

1% aqueous potassium gluconate*	-----	1 part
2.5% potassium persulphate*	-----	1 part
Modified dilute Nessler's reagent	-----	2 parts

Eastman Kodak Wratten Gelatin Filter No. 75.—

Technic.—

Place in a 250 c.c. Erlenmeyer flask 5 c.c. of whole oxalated blood.

Add 35 c.c. distilled water.

Add 10 to 20 mg. of urease powder and mix well.

Allow to stand at room temperature for 20 minutes. Room temperature must not be below 20° C.

Add 5 c.c. of 10% sodium tungstate solution and mix.

Add 5 c.c. of 0.66 N sulphuric acid, shaking vigorously during the addition, shake, and let stand for 10 minutes.

Filter through Whatman No. 2 filter paper. After filtration has proceeded for 10 minutes, pour the filtrate back onto the paper, and continue the filtration.

Into a test tube graduated at 20 c.c. and 25 c.c., marked "U" for unknown, place 5 c.c. of the filtrate.

Into a similar test tube marked "S" for standard, place 5 c.c. of the standard working solution of nitrogen (5 c.c. contain 0.075 mg. nitrogen).

Add distilled water to each tube to the 20 c.c. mark.

Mix thoroughly.

Add to each tube 4 c.c. of the Nessler's mixture, freshly made, and dilute at once with distilled water to the 25 c.c. mark.

*By using 1% sodium citrate, the potassium gluconate and potassium persulphate can be omitted.

¹J. Biol. Chem. 143: 2, April, 1942.

Stopper the test tubes, mix vigorously, let stand for 15 minutes to develop the full color, and compare in the colorimeter.

Colorimeter.—

It is preferable, when using a visual colorimeter, to place a small filter disc in the eyepiece of the colorimeter which will give sharp definition in the comparison of the resulting colors. Eastman Kodak Wratten gelatin filter, number 75, is recommended.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 15 = \text{mg. of urea nitrogen per 100 c.c. of blood.}$$

To convert from urea nitrogen to urea multiply the results by 2.14.

If a photoelectric colorimeter is used, insert the proper filter, secure full-scale deflection with a distilled water blank, and read the standard and then the unknown. This method is better than using a calibration curve, since the color varies greatly with temperature and with the age and lot of the reagents.

Calculation for Direct Reading Photoelectric Colorimeters.—

$$\frac{\text{Reading of Unknown}}{\text{Reading of Standard}} \times 15 = \text{mg. urea N per 100 c.c. blood.}$$

With colorimeters' reading per cent of transmission only, the density must be calculated. This is the log of (1/transmission) or $\log 1 - \log T$. Since scale readings are in terms of per cent of transmission, the above becomes $\log 100 - \log \text{ of reading}$, or $2 - \log \text{ of reading} = \text{density } (D)$. Values for D for standard and unknown are then substituted in the formula:

$$\frac{D \text{ Unknown}}{D \text{ Standard}} \times 15 = \text{mg. urea N per 100 c.c. blood.}$$

Normal:

Urea nitrogen, 10 to 15 mg. per 100 c.c. blood.
Urea, 25 to 30 mg. per 100 c.c. blood.

Micromethod for Blood Urea Nitrogen, Keller¹

Reagents.—

Tungstic Acid.—

To 80 c.c. of distilled water add 16 c.c. of N/12 sulphuric acid volumetric solution. Mix and add 2 c.c. of 10% sodium tungstate solution. (The sodium tungstate is of special grade.)

Urease Solution.—

Place 15 gm. of jack bean meal and 5 gm. of permutit in an Erlenmeyer flask. Add a mixture of 16 c.c. of alcohol and 84 c.c. of distilled water. Shake continuously for 10 minutes, then allow to stand for 18 hours in a refrigerator. Filter and keep in a refrigerator.

Phosphate Buffer.—

Dissolve 14 gm. of sodium pyrophosphate and 2 gm. of metaphosphoric acid in water and dilute to 250 c.c.

Urea Stock Solution.—

Dissolve 0.1286 gm. of urea in water and dilute to 200 c.c.

Urea Standard Solution.—

Dilute 5 c.c. of the stock urea solution to 100 c.c. with distilled water. (5 c.c. is equivalent to 0.075 mg. N).

Nessler's Solution (see page 244).—

¹Keller, A. G., Jr.: J. Lab. & Clin. Med. 17: 1146, 1932.

Equipment.—

- 1 15 c.c. conical centrifuge tube.
- 1 10 c.c. graduated pipette.
- 1 Special microblood pipette.
- 1 2 c.c. volumetric pipette.
- 2 1 c.c. pipettes graduated in 0.1 c.c.
- 1 3 c.c. volumetric pipette.
- Water-bath heated to 50° C.
- 2 small test tubes.
- 2 test tubes graduated at 9 and 10 c.c.

Technic.—

Place 9.8 c.c. of the tungstic acid solution in a 15 c.c. conical centrifuge tube.
 Draw 0.2 c.c. blood from ear or finger prick.
 Discharge 0.2 c.c. blood into the tungstic acid solution.
 Rinse the pipette with the solution.
 Stopper tube and mix thoroughly.
 Allow to stand a few minutes and centrifuge.
 Transfer 4 c.c. of the supernatant fluid to a small test tube and label "U" for unknown.
 Into a similar test tube labeled "S" for standard, pipette 1 c.c. of urea nitrogen standard solution (containing 0.015 mg. N).
 Add 3 c.c. distilled water.
 To "U" and "S" add 3 drops of urease and 3 drops of the buffer solution.
 Incubate both tubes in the water bath heated to 50° C. for 10 minutes.
 At the end of this time transfer the contents of the tubes to graduated tubes similarly marked for the unknown and standard.
 Rinse the tubes with distilled water and dilute to the 9 c.c. graduation.
 Add to both tubes 1 c.c. of Nessler's solution and compare colorimetrically.

Calculation.—

If the **unknown** is set at 15 mm., the reading of the standard multiplied by 1.25 gives the mg. of urea nitrogen per 100 c.c. of blood;

or use the formula
$$\frac{S}{R} \times 0.015 \times \frac{100}{0.08}$$

Example.—

Standard matches at 10.

$$10 \times 1.25 = 12.5 \text{ mg. urea nitrogen in 100 c.c. blood.}$$

Normal is 10 to 15 mg. urea nitrogen, or 25 to 30 mg. of urea, in 100 c.c. blood. To convert to urea, multiply the urea nitrogen figures by 2.14.

Titrimetric Ultramicromethod for Urea and Kjeldahl Nitrogen Micromethod of Sobel, Mayer, and Gottfried^{1, 2}

This is an aeration titration method requiring a minimum of manipulation. The method is adapted to the estimation of urea in 0.1 c.c. of blood serum containing 5 to 100 γ of urea N and for the estimation of total N, albumin N, and nonprotein N with 0.01 to 0.05 c.c. of blood serum.

Reagents.—**Phosphate Buffer pH 7.0.—**

Dissolve 15 gm. of sodium pyrophosphate
 and 2 gm. of phosphoric acid (1.4 c.c. of 85% H₃PO₄)
 in 100 c.c. of distilled water.

¹Sobel, A. E., Mayer, A. M., and Gottfried, S. P.: J. Biol. Chem. **156**: 355, 1944.

²Sobel, A. E., Hirschman, A., and Besman, L.: J. Biol. Chem. **161**: 99, 1945.

Urease Extract.—

Place 10 gm. of washed permutit in a liter Erlenmeyer flask. Wash the permutit with three 50 c.c. portions of 2 per cent acetic acid; then add 100 c.c. of 2 per cent acetic acid.

Add 150 c.c. of 0.001 N sulphuric acid and 50 gm. of jack bean meal. Shake for 15 minutes.

Add 200 c.c. of glycerol and again shake for about 10 minutes; then place in refrigerator. Stir the mixture occasionally by shaking, and allow to settle overnight in the refrigerator.

Remove the supernatant fluid and centrifuge for 1 hour at 2,000 r.p.m.

Transfer the clear supernatant liquid to a bottle, and keep in the refrigerator. The extract so prepared is very stable. One may also use commercially prepared urease tablets containing a buffer.

Indicator.—

Mix 8 parts of 0.1 per cent bromocresol green in 95 per cent alcohol, and 1 part of 0.1 per cent methyl red in 95 per cent alcohol.

2 Per Cent Boric Acid With Indicator.—

Dissolve 20 gm. of boric acid in distilled water and dilute to 1 liter with distilled water.

Mix 100 parts of the 2 per cent boric acid with 1 part of the indicator.

Urea Nitrogen Recovery Solution.—

Dissolve 2.1434 gm. of recrystallized urea in distilled water and dilute to 1,000 c.c. in a liter volumetric flask. 1 c.c. contains 1 mg. of urea nitrogen.

Urea Nitrogen Standard Solution.—

Dilute 10 c.c. of urea nitrogen recovery solution to exactly 100 c.c. with distilled water in a 100 c.c. volumetric flask. 1 c.c. contains 0.1 mg. of urea nitrogen.

Ammonia-Free Caprylic Alcohol Saturated with Thymol.—(Antifoam Reagent)**Potassium Carbonate, Half Saturated Solution.—**

Use approximately 560 gm. of anhydrous potassium carbonate per liter.

Standardized 0.0714 N Sulphuric Acid.—

Dilute 71.4 c.c. of N/1 sulphuric acid (page 24) to exactly 1,000 c.c. with distilled water in a liter volumetric flask, and titrate against a standard alkali.

Digestion Mixture.—

In 500 c.c. of distilled water, place

30 gm. of potassium sulphate

and 5 gm. of copper sulphate.

Dissolve and add 480 c.c. of concentrated sulphuric acid

and 0.5 gm. of selenium in 20 c.c. of concentrated sulphuric acid.

5 Per Cent Trichloroacetic Acid.—

Dissolve 5 gm. of trichloroacetic acid, analytical reagent grade, in enough distilled water to make 100 c.c. of solution.

23 Per Cent Sodium Sulphate Solution.—

Dissolve 23 gm. of sodium sulphate, c. p., in enough distilled water to make 100 c.c. solution. Keep in an incubator at 37° C.

Alkali Solution.—

Dissolve 60 gm. of sodium hydroxide

and 10 gm. of sodium thiosulphate

in 120 c.c. of distilled water.

Ether, U.S.P.—**Standard and Recovery Solution for Proteins.—****Stock Solution.—**

Dissolve 4.7186 gm. of ammonium sulphate, which has been dried overnight at 105°, in 100 c.c. of distilled water. 1 c.c. equals 10 mg. N.

Solution for Use.—

Place 1 c.c. of the stock solution in a 100 c.c. volumetric flask and dilute to 100 c.c. with distilled water. 1 c.c. equals 0.1 mg. N.

Equipment.—

Special microaeration test tubes with side arm (12 cm. long and 1.5 cm. in diameter). (Made by Emil Greiner Company, 161 Sixth Ave., New York 13, N. Y.)

Glass tubing, 15 to 18 cm. long, 4 mm. outside diameter, with drawn out fine tip of about 1 to 1.5 mm. diameter.

1-hole, size 0, rubber stoppers.

Rubber tubing, about 33 cm. long, 8 mm. outside diameter, and 3.5 mm. inside diameter.

Capillary microburet (Rehberg, self-filling type).

Hot-plate.

Electric pump or water suction.

Aeration and Titration.—(Fig. 103.)

Connect the digestion tube (tube 2) of the aeration outfit, containing 10 or 100 γ of ammonia nitrogen and 1 c.c. of distilled water, with a rubber tubing to the receiving tube (tube 1), which contains 1.5 c.c. of the boric acid solution containing the indicator.

Connect the other outlet of the digestion tube with a tube containing boric acid and indicator. This serves as a trap for the ammonia in the air. See Fig. 103.

Add to the digestion tube 0.5 c.c. of the alkali solution for Kjeldahl N, stopper the tubes immediately, and begin the aeration slowly. After 20 to 30 minutes the ammonia is completely aerated over and trapped in the boric acid. Stop the aeration by rapidly disconnecting the tubes from the pump. While the glass tubes, which are inserted into the receiving tubes, are being removed, carefully wash them down with a few drops of distilled water.

At the pH of the boric acid (4.2) the indicator is a faint pink, which turns blue at a more alkaline pH, in this case caused by the ammonia. With the 0.0714 N sulphuric acid, titrate the boric acid solution back to its original pH with the aid of a capillary microburet, keeping the tip of the buret beneath the surface of the liquid, and stirring by means of a stream of air during this titration. A similar receiving tube containing the boric acid and indicator mixture serves for matching the color of the end point.

Technic for Micro-Kjeldahl Analysis.—

Add 0.2 c.c. of digestion mixture to the sample to be analyzed, or to 0.1 c.c. of standard ammonium sulphate in a control analysis, in a digestion tube. Digest on a hot-plate. After it has cooled for a few moments, wash down the walls of the test tube with 1.0 c.c. of distilled water. From here on the procedure of aeration and titration is continued as described.

Nonprotein Nitrogen.—

Precipitate the protein of 0.1 c.c. of serum with 2.5 c.c. of trichloroacetic acid, and centrifuge for 10 minutes at 2,000 r.p.m.

Digest a 1.0 c.c. aliquot with 0.2 c.c. of digestion mixture.

After it has cooled for a few moments, add 1.0 c.c. of distilled water and proceed with the aeration and titration as above.

Calculation.—

Titration \times 2,600 = mg. per cent of nonprotein nitrogen.

Normal Nonprotein Nitrogen = 25-35 mg. per 100 c.c. blood.

Total Protein (Total Nitrogen).—

Place 0.1 c.c. of serum in a test tube and dilute to 1 c.c. with distilled water.

Digest 0.1 c.c. of this mixture with 0.2 c.c. of digestion mixture on a hot plate.

Place the tubes at an angle of about 45° and take care that none of the liquid bumps out. Digestion is completed when the liquid turns colorless or bluish green. Allow to cool for a few moments and add 1 c.c. of distilled water to wash down the walls of the test tube.

Carry out the aeration and titration exactly as described for known ammonium salts in the presence of the digestion mixture.

A reagent blank must be run simultaneously.

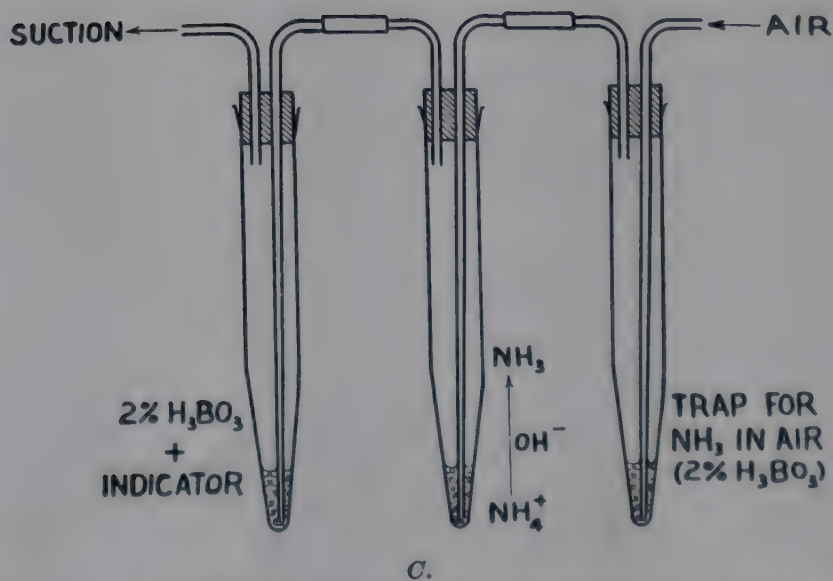
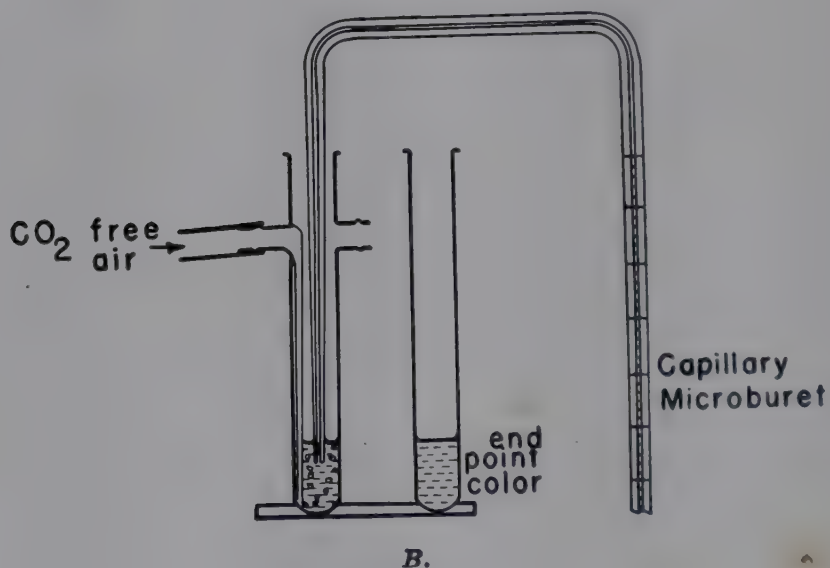
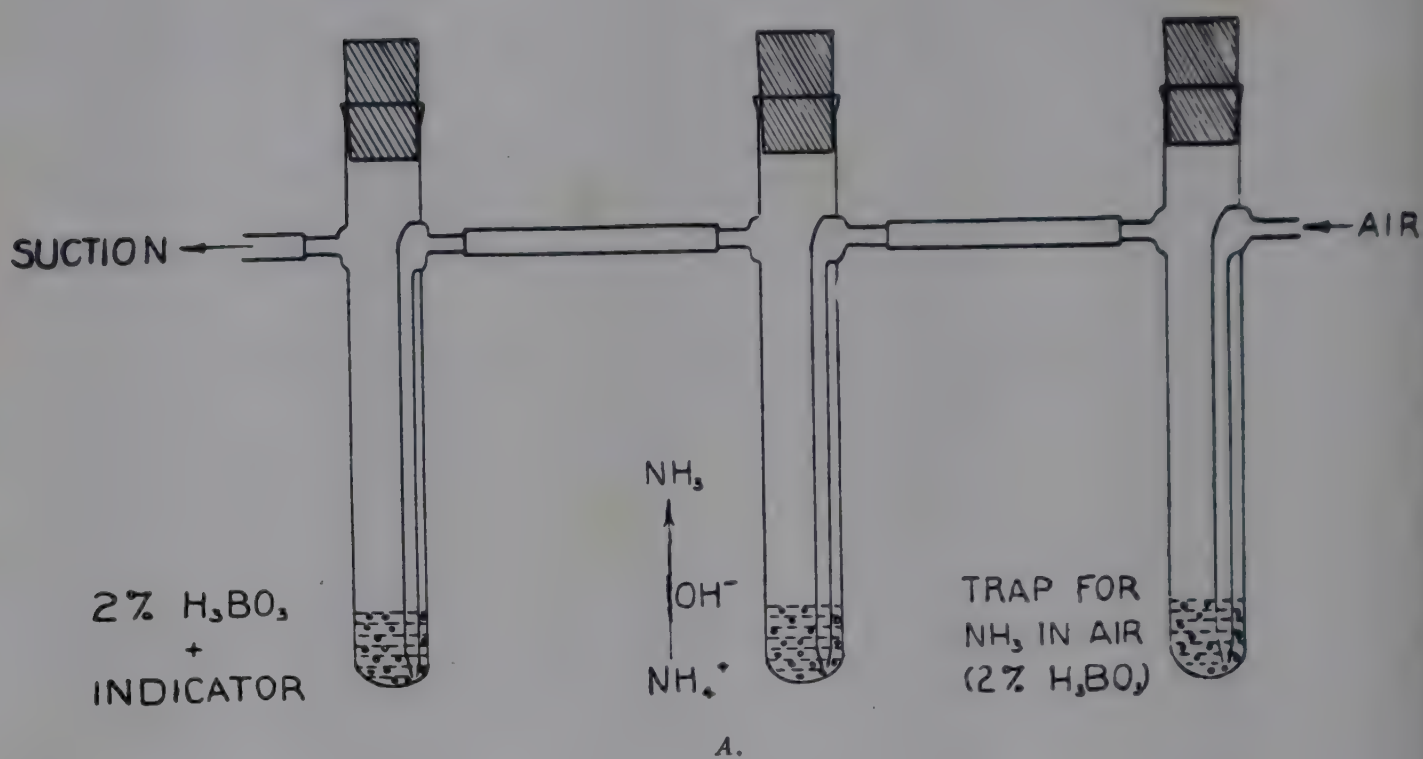


Fig. 103, A, B, C.—A, schematic diagram of microaeration apparatus for 10 to 200 micrograms of N: Tube 1 (receiving tube), tube 2 (digestion tube), and trap. B, Titration assembly. C, Schematic diagram of microaeration apparatus for 1 to 10 micrograms of N. (From Sobel.)

Calculation.—

Total nitrogen: c.c. of titration $\times 0.0714 \times 14$ = amount of N in sample; c.c. of titration $\times 10,000$ = mg. per cent of Kjeldahl N.

Normal Serum Total Protein = 6.5 to 8.2 per cent.

Total Protein (mg. per cent of total N minus mg. per cent of nonprotein N) $\times 6.25$ divided by 1000 = gm. per cent of total protein.

Albumin.—

Precipitate the globulin of 0.2 c.c. of diluted serum (0.1 in 1.0 c.c.) with 0.3 c.c. of sodium sulphate in a small test tube (100 \times 12 mm.) and shake up with 1 c.c. of ether for 1 to 2 minutes.

Centrifuge for 10 minutes at 2,000 r.p.m.

Digest 0.2 c.c. aliquots of the aqueous layer with 0.2 c.c. of digestion mixture.

Determine the Kjeldahl nitrogen as described above.

Calculation.—

Titration $\times 12,500$ = mg. per cent of albumin N + nonprotein N; (mg. per cent of albumin N + nonprotein N minus mg. per cent of nonprotein N) $\times 6.25$ divided by 1000 = gm. per cent of albumin.

Normal Serum Albumin = 4.6 to 6.7 per cent.

Urea Nitrogen**Technic.—**

Add 0.1 c.c. of serum, spinal fluid, or urine, providing a sample with 10 to 200 γ of urea N, to the contents of a digestion tube which consists of 1 drop of phosphate buffer, 1 drop of urease extract, and 3 drops of antifoam reagent.

After an incubation period of 10 minutes, add 0.5 c.c. of potassium carbonate and aerate the ammonia for 20 to 30 minutes. Urines are aerated a little longer because of the high values of urea. Otherwise, first dilute 0.1 c.c. of urine to 1 c.c. with distilled water, then treat 0.1 c.c. of the mixture in the same manner as serum.

For the titration, use a capillary microburet with 0.0714 N sulphuric acid to simplify the calculation.

Calculation.—

Mg. of urea N per 100 c.c. of sample = c.c. of acid used in titration $\times 1000$ when 0.0714 N acid is employed.

To convert to urea, multiply by 2.14.

In these methods it is important to remove impurities from newly obtained rubber stoppers and tubing, which can be accomplished by boiling in a dilute solution of sodium hydroxide followed by washing with distilled water and finally with 2 per cent boric acid.

Note that the new antifoaming mixture, caprylic alcohol saturated with thymol, employed for the urea estimation, probably works because the thymol alters the surface properties of the proteins in addition to the usual behavior of caprylic alcohol in increasing interfacial tension. Caprylic alcohol alone does not reduce foaming sufficiently to be useful.

The titration involves no special precautions. A titration with a capillary microburet takes no more time than with an ordinary buret. Both the buret tip and the stirring tip are inserted into the liquid to be titrated.

Normal

Urea N = 10-15 mg. per 100 c.c. blood

Urea = 25-30 mg. per 100 c.c. blood.

Urea Nitrogen, Direct Nesslerization* Method of Karr**Principle.¹—**

By the action of urease, the urea in the filtrate is converted to ammonium carbonate which is nesslerized in the presence of gum ghatti as a protective colloid.¹ The interference

*Hawk and Bergeim: Practical Physiological Chemistry, ed. 11, P. Blakiston's Son and Co., Inc., p. 423.

¹Karr: J. Lab. & Clin. Med., 9: 3, 1924. The use of gum ghatti as a stabilizing colloid by Folin (J. Biol. Chem. 81: 231, 1920), in his sugar method, suggested its application in the urea method to Looney (J. Biol. Chem. 88: 189, 1930).

of peptones and amino acids (Folin and Wu) is regarded as so slight and uniform as not to influence the clinical value of the results. This method offers the practical advantages of speed and the possibility of performing many determinations at one time.

Reagents.—

2/3 Normal Sulphuric Acid.—See page 225.

10% Sodium Tungstate Solution.—See page 225.

Nessler's Solution.—This is the same as that used in Folin and Wu nonprotein nitrogen, page 244.

Buffer Solution.—

Prepare first, half normal phosphoric acid by diluting 17 c.c. of 85% phosphoric acid to one liter with distilled water, in a liter volumetric flask. Titrate 5 c.c. of this with N/10 alkali, using phenolphthalein as an indicator, until a faint pink color develops. On the basis of this titration, dilute the acid to a substantially correct half normal solution.

Dissolve 14 grams of sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$) in enough half normal phosphoric acid to make 100 c.c., using a 100 c.c. volumetric flask. The pH of the buffered filtrate should not be less than 4.0.

Urease Solution.—

Transfer 0.5 gram of jack bean meal to a clean 50 c.c. flask.

Add 20 c.c. of 30% (by volume) alcohol.

Shake for 10 minutes and filter or centrifuge.

This extract should always be prepared on the day it is to be used, because on standing even in a refrigerator it will develop ammonia and will yield too high results. One should, therefore, not use more extract or a stronger extract than is really necessary.

Stock Urea Solution.—

This is the same as used in the Keller micromethod for urea nitrogen, page 252.

Standard Urea Solution.—

This is the same as used in the Keller micromethod for urea nitrogen, page 252.

Gum Ghatti Solution.—

This is the same as that given in the Folin and Wu method for total nonprotein nitrogen, page 245.

Technic.—

Into a test tube marked "U" for unknown, place 5 c.c. of the Folin-Wu blood filtrate (page 224).

Into a tube marked "S" for standard, place 5 c.c. of the standard urea solution.

To each tube add one drop of buffer solution and either a strip of urease paper or 5 drops of urease solution.*

Place the tubes in a water bath at 50° C. for 15 minutes.

Transfer contents of both tubes, with rinsings, to test tubes marked respectively "U" and "S," and graduated at 22.5 and 25 c.c.¹

Dilute to the 22.5 c.c. mark with distilled water.

Add 3 drops of gum ghatti solution to each tube.

Add Nessler solution to the 25 c.c. mark.

Mix the solutions and read about one minute later in a colorimeter.

In the case of blood filtrates high in urea N (40 mg., or over, per 100 c.c. of blood), repeat the determination, using smaller amounts of filtrate.

In laboratories where many determinations of blood urea N are conducted daily it is advisable to use an artificial permanent standard and control it by daily checking against the standard urea solution.

*See note under Urease Solution on page 247.

¹Separate tubes for nesslerization are necessary because of the "poisoning" action of mercury on urease. When this is suspected in the conversion tubes, they should be cleaned with strong nitric acid. Instead of graduated test tubes, ordinary 25 c.c. graduated cylinders may be used.

Calculation.—

Set the unknown on the left side of the colorimeter at exactly 15 mm. Read the standard against this unknown. The reading of the standard then gives directly the urea N per 100 c.c. of blood. If less than 5 c.c. of filtrate (0.5 c.c. blood) are used, make the necessary correction by using the following formula and substituting the amount of blood in the filtrate used for the 0.5 in the formula.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.075 \times \frac{100}{0.5} = \text{urea N per 100 c.c. blood.}$$

Normal Urea Nitrogen.—10 to 15 mg. per 100 c.c. of blood. To convert to urea, multiply by 2.14. **Normal Urea**—25 to 30 mg. per 100 c.c. blood.

Blood Urea Nitrogen, Xanthydrol Reaction of Goldbaum¹

One of the best methods for direct determination of urea in blood is to precipitate it with xanthydrol and then estimate the resulting dixanthhydril urea. Fosse first showed that two molecules of xanthydrol combine with urea to form a ureide the weight of which is seven times that of urea. With others, he applied this reaction for the quantitative determination of small amounts of urea in blood, urine, and tissues.

Numerous procedures have been used for estimating dixanthhydril urea. These methods require special apparatus and are time consuming and expensive to manipulate. They are not suited, therefore, for routine determinations of urea if large numbers of blood specimens are to be examined.

The Goldbaum method is a simple procedure for the determination of urea utilizing the xanthydrol precipitation principle for routine blood and urine urea analyses.

Reagents.—**Stock Xanthydrol Solution.—**

Add 10 gm. xanthydrol, Eastman Kodak, to 100 c.c. of absolute methyl alcohol. Shake the mixture well and filter off the insoluble material. The clear, slightly yellow solution will keep for weeks if kept in a dark brown bottle.

Working Solution of Xanthydrol.—

Prepare this solution fresh for each analysis.

Add 5 c.c. of the stock xanthydrol solution to glacial acetic acid in a 50 c.c. volumetric flask, and dilute to 50 c.c. with glacial acetic acid.

10% Sodium Tungstate and 2/3 N Sulphuric Acid.—

These are the same as those used in the preparation of Folin protein-free filtrate, page 224.

Stock Solution of Urea.—

Dissolve 214.3 mg. of urea, reagent grade, in distilled water in a 100 c.c. volumetric flask, and dilute to 100 c.c. with distilled water.

Each cubic centimeter of this solution contains 2.143 mg. of urea, or 1 mg. of urea nitrogen.

Standard Solution of Urea.—

Add 5 c.c. of the stock solution of urea to distilled water in a 200 c.c. volumetric flask and dilute to 200 c.c. with distilled water.

1 c.c. contains 0.0536 mg. of urea, or 0.025 mg. of urea nitrogen.

Pyrex Constable Protein Centrifuge Tubes.—

These are 15 c.c. tubes with capillary stems graduated from 0 to 0.1 c.c. in 0.005 c.c. divisions. The upper section is graduated at 1, 2, 3, 4, 5, and 10 c.c.

¹Goldbaum, L.: Personal communication.

Technic.—**Standardization of the Constable Centrifuge Tubes.—**

Place in the centrifuge tubes 2 c.c., 1 c.c., and 0.5 c.c., respectively, of the standard solution of urea containing 0.0536 mg. of urea per c.c. 2 c.c. contain 0.1072 mg. urea or 0.05 mg. urea N; 1 c.c. contains 0.0536 mg. urea, or 0.025 mg. urea N; and 0.5 c.c. contains 0.0268 mg. urea or 0.0125 mg. urea N.

Add sufficient distilled water to bring the volume in each tube to 3 c.c.

Add 3 c.c. of freshly prepared acetic acid xanthidrol solution to each tube. Mix the contents of each tube by means of a glass rod fine enough to enter the capillary stem of the centrifuge tube.

Allow to stand for 5 minutes. Centrifuge at 2500 r.p.m. for 5 minutes.

Read off the number of divisions on the tube at which the precipitate column stands. Convert to urea or urea nitrogen. Each 0.005 division represents $\frac{\text{urea or urea N in the standard}}{\text{number of divisions}}$ or X mg. of urea or urea N.

Example.—2 c.c. of standard urea solution were used. This represents 0.1072 mg. urea or 0.05 mg. urea N. The precipitate stood at 8 divisions (0.04 c.c.). $0.1072 \div 8 = 0.0134$ mg. urea. Or $0.05 \div 8 = 0.0063$ mg. urea N. The information derived from this standardization procedure is used in the final calculation of results of the test.

Test on Blood

Prepare a Folin protein-free blood filtrate (page 224).

Place 3 c.c. of this filtrate in a Constable centrifuge tube.

Determine the urea or urea nitrogen using the technic described under standardization of the Constable centrifuge tubes, above.

If the concentration of urea exceeds 45 mg. in 100 c.c. of blood, the precipitate tends to accumulate at the top of the capillary stem of the tubes. If this occurs, repeat the determination, using 1 or 2 c.c. of the filtrate and dilute it to 3 c.c. with distilled water. Make proper corrections in the calculations.

Calculations.—

Let *a* represent the volume of the unknown precipitate in terms of 0.005 c.c. divisions on the Constable tube.

Let *b* represent the urea or urea N equivalent of each division as determined in the standardization of the Constable tubes.

Let *c* represent the volume of filtrate used in the test.

$$a \times b \times \frac{1000}{c} = \text{mg. of urea or urea N in 100 c.c. of blood.}$$

Example.—

Unknown precipitate stood at 0.030, or 6 divisions.

1 division (see above) represented 0.0134 mg. urea or 0.0063 mg. of urea N.

$$6 \times 0.0134 \times \frac{1000}{3} = 26.8 \text{ mg. urea per 100 c.c. blood.}$$

$$6 \times 0.0063 \times \frac{1000}{3} = 12.6 \text{ mg. urea N per 100 c.c. blood.}$$

Normal Urea Nitrogen.—10-15 mg. urea nitrogen per 100 c.c. of blood.

To convert urea nitrogen to urea multiply by 2.14.

Normal Urea.—25-30 mg. per 100 c.c. of blood.

Test on Urine

This method is applicable to the examination of urine for urea content. The presence of ammonia in the urine does not interfere with the determination of urea by the xanthidrol reaction.

Dilute 1 c.c. of urine to 500 c.c. with distilled water, using volumetric glassware.

Dilute an aliquot of 1 c.c. of the diluted urine to 3 c.c. with distilled water.

Carry out the test in the same manner as the examination of blood.

If the concentration of urea in the specimen is either too great or too small, repeat the test using a different aliquot.

Calculations.—

a and *b* represent the same as in the test for urea in blood (above).

Let *c* represent the volume of diluted urine used in the test.

$$a \times b \times \frac{50,000}{c} = \text{mg. urea (or urea N) in 100 c.c. of urine.}$$

Normal Urea.—10 to 40 gm. in 24 hours.

LaMotte Blood Urea Outfit¹

The LaMotte blood urea outfit utilizes a simple and rapid procedure for testing for urea. This is based on the mercury combining power of the blood, which is an accurate index of the retention of nitrogen and especially urea. The blood is deproteinized with trichloroacetic acid solution, and the filtrate is titrated with standard mercuric chloride solution employing an outside indicator to determine the end point. Only 15 to 20 minutes are required to make a complete estimation of blood urea. The simplified technic contributes greatly to obtaining reproducible results.

Urea Clearance Tests

This test is used to determine the volume of blood which is entirely cleared of urea in one minute or the volume which would be so cleared if all the urea excreted in one minute is abstracted entirely from one portion of blood. The amount of urea excreted per minute varies directly with the concentration of urea in the blood. With large volumes of urine the rate of urea elimination is directly proportional to the blood urea content. If the urine excretion is over 2 c.c. per minute, we call this the "augmentation volume." If the urine excretion falls below 2 c.c. per minute, the urea excretion rate falls and is proportional to the square root of the urine volume in cubic centimeters per minute. The percentage of urea in the urine is quite constant under normal conditions, even though the quantity of urea eliminated varies with the protein intake. In determining the urea clearance three factors are considered: (1) the concentration of urea in the blood, (2) the urea concentration in the urine, and (3) the volume of urine excreted in the unit of time. (See Ambard Coefficient, page 155.)

Significance of Urea Clearance Figures.—(See pages 162 and 176.)

Normally there is a variation of urea clearance from 75 to 130 per cent. This variation decreases as urea clearance decreases such as is seen in nephritis. Figures over 75 indicate unimpaired function; values from 75 to 50 are doubtful; and under 50 point to renal impairment.

Urea clearance may be affected by factors other than renal. Cardiac disease, with slowing of the circulation within the body and within the kidney, coexisting with the renal impairment, will produce a definite change in the clearance. Other factors may be dehydration (persistent vomiting or diarrhea), shock, reflex anuria of nervous origin, and back pressure from ureteral, bladder, or prostatic pressure.

In acute nephritis, the urea clearance may be normal or may be considerably below normal. This fall is not necessarily a bad omen unless it fails to return to a higher figure within four months after the onset of the renal lesion. If this rise does not occur within this time period we may conclude that the acute condition is becoming chronic.

¹Hench, P. S., and Aldrich, Martha: Arch. Int. Med. 38: 474-488, 1926.

In chronic nephritis the clearance is proportional to the amount of destruction in the glomerular structures. Regarding low figures in chronic nephritis, a figure below 10 indicates impending uremia.

It is generally believed that a clearance that definitely remains as low as 20 per cent or lower will eventuate in death within two years. Uremia is always present with values as low as 5 and is uniformly absent with values above 10.

Elmer Belt, of Los Angeles, California, published, in his article on modern tests of kidney function,¹ a chart showing the correlation of the principal renal function tests. This shows in an extremely interesting fashion the comparative values of the various tests. See Fig. 77, page 176.

Blood Urea Clearance

Method of Van Slyke and Cape, Simplified by Pons²

Reagents.—

N/12 Sulphuric Acid.—

10% Solution of Sodium Tungstate.—

This is the same solution that is used for the preparation of Folin protein-free filtrate. (Page 225.)

Urease Paper.—

To prepare urease paper 15 gm. of jack bean meal is shaken up with 5 gm. of permutit in 100 c.c. of 15% alcohol for 15 minutes using a mechanical shaker or for one-half hour if shaken by hand. Filtration is carried on in the refrigerator overnight. Pieces of heavy, ammonia-free filter paper are drawn through the solution and hung over cord to dry. The paper is cut in pieces 1 by 1 inch.

Phosphate Buffer Solution.—

Dissolve 14 grams of sodium pyrophosphate in enough N/2 phosphoric acid to make 100 c.c. The N/2 phosphoric acid is made by diluting 20 c.c. of 85% phosphoric acid to 1 liter and titrating 5 c.c. with N/10 alkali, using phenolphthalein as an indicator. A faint pink is the end point. Correct as needed.

Nessler's Reagent.—

This is the same reagent that is used for the determination of nonprotein nitrogen. (Page 244.)

Permutit.—

Equipment.—

- 2 10 c.c. volumetric pipettes.
- 1 4 inch funnel.
- Filter paper.
- 4 large test tubes (1 × 8 inches).
- 1 3 c.c. volumetric pipette.
- 3 1 c.c. graduated pipettes.
- 1 2 c.c. volumetric pipette.
- 2 5 c.c. volumetric pipettes.

Principle.—

The urine is diluted to such an extent that if the clearance, either standard or maximum, is the average for a normal subject, the urea concentrations in the urine or diluted blood will be equal. The urea in both blood and urine is converted to ammonia with urease,

¹Vol. II, Cabot's *Modern Urology*, issued by Lea & Febiger, Philadelphia.

²Pons, C. A.: *Am. J. Clin. Path.* 8: 33, 1938.

proteins are removed, and the ammonia contents of the two filtrates are compared colorimetrically. A single reading gives directly the average normal renal function in terms of the clearance.

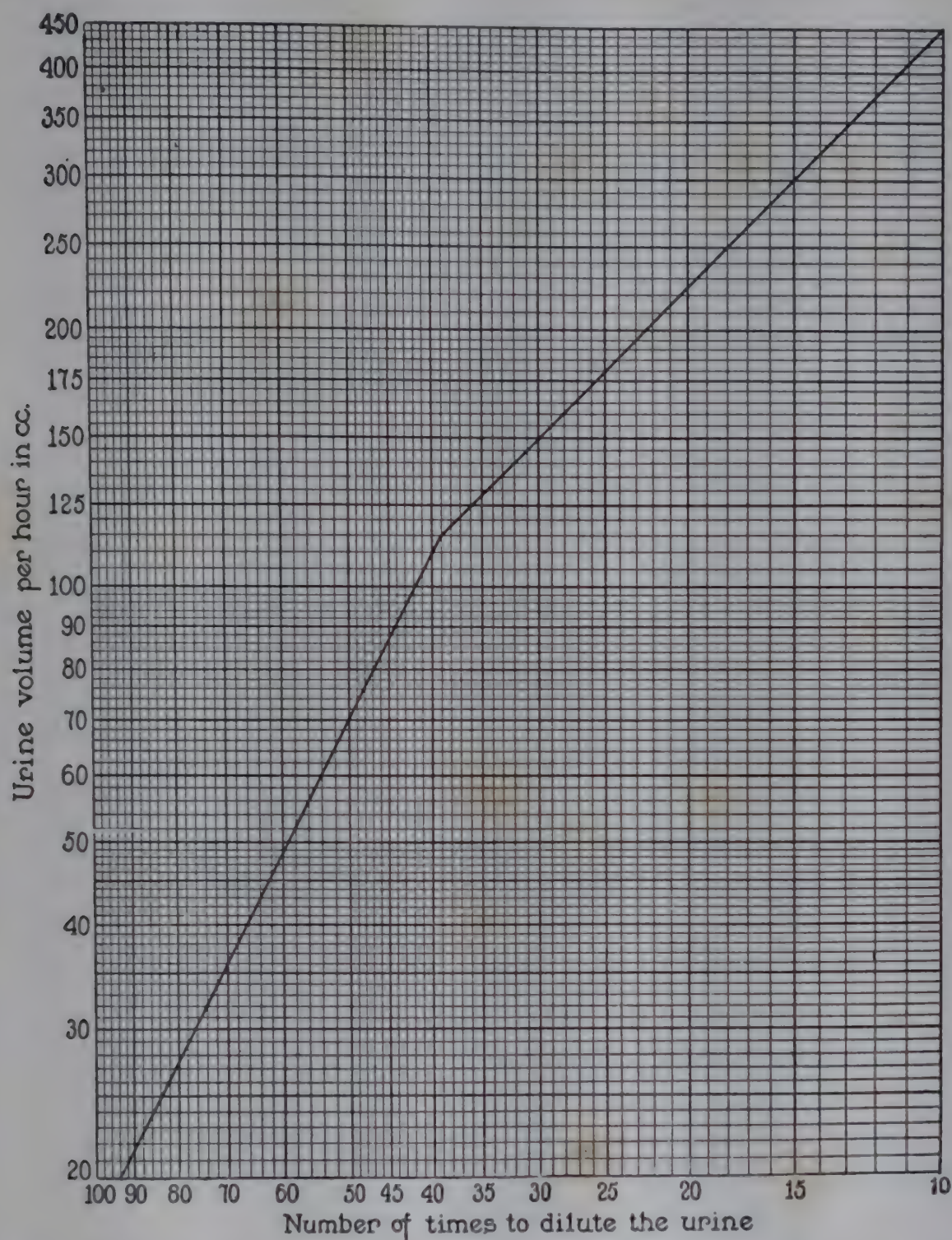


Fig. 104.—Urine dilution chart. (After Peters and Van Slyke: *Am. J. Clin. Path.*, 1938.)

Technic.—

A few minutes before the test the patient is given a glass of water and asked to empty the bladder. After one hour the urine sample is collected and a sample of blood is drawn. One hour later a second urine sample is collected. The two specimens of urine are carefully measured and mixed. The timing of the collections of urine must be exact and if necessary the patient must be catheterized in order to be sure that there is no residual urine in the bladder.

Place 10 c.c. of the urine in a large test tube.

Add 2 grams of permutit, shake at frequent intervals for 10 minutes, and filter. (If the urine shows more than a heavy trace of albumin add 2.5 gm. of permutit instead of the usual 2 gm.)

Place 2.0 c.c. in a large test tube.

Add 3.2 c.c. of N/12 sulphuric acid.

Add 14.4 c.c. of distilled water, and allow to stand for a few minutes.

Add 0.4 c.c. of 10% sodium tungstate solution, and allow to stand a few minutes.

Filter.

After the number of c.c. of urine excreted in an hour is estimated, the number of times that 1.0 c.c. of the urine filtrate is to be diluted can be obtained by consulting the dilution chart (Fig. 104). For example, if the urine volume is 80 c.c. per hour, the urine according to the chart should be diluted 46.8 times. If, however, the subject is a child of 1.4 meters' height, the 80 c.c. observed hour's volume is multiplied by the factor 1.49. The corrected volume 119 c.c. is then interpolated on the curve of the chart. In this case the dilution should be only 37.5 times.

To a large test tube, graduated at 22.5 c.c. and 25 c.c., add 5 c.c. of the diluted urine filtrate.

To a similar tube add 5 c.c. of Folin protein-free blood filtrate. (Page 224.)

Add 2 strips of urease paper to each tube.

Add 3 drops of phosphate buffer to each tube.

Incubate at 45 to 50° C. for 15 minutes.

Dilute the contents of both tubes to 22.5 c.c. with distilled water.

Dilute the contents of both tubes to 25 c.c. with Nessler's reagent.

Compare in the colorimeter, setting the test on the blood filtrate specimen in the standard cup and the urine specimen test in the unknown cup.

Calculation.—

$$\frac{100 \times \text{Reading of blood filtrate}}{\text{Reading of urine filtrate}} = \text{percentage of average normal clearance.}$$

If the clearance is less than half the normal average, the blood filtrate when nesslerized will be more than twice the color of the urine filtrate, then a fresh portion of the blood filtrate is diluted two, three, five, or ten times in order to bring the blood-urine ratio to about the same color. In such instances the calculation becomes:

$$\frac{100 \times \text{Reading of blood filtrate}}{\text{Dilution of blood} \times U} = \text{percentage of average normal clearance.}$$

U = reading of urine filtrate.

Blood Urea Clearance Test—Möller, McIntosh, and Van Slyke¹

This method is based upon measurement of the excretory efficiency of the kidneys by the amount of blood cleared of urea in one minute as determined by the ratio of the blood urea to the amount of urea excreted in the urine during a fixed time.

Technic.—

The test is carried out during the period when excretion is least likely to fluctuate, namely, between breakfast and luncheon. It is a good procedure to have the patient eat his breakfast at 8 A.M. without tea or coffee. At 9 A.M. the bladder is completely emptied, and the urine specimen is discarded. Patient is given 100 c.c. of water. At 10 A.M. bladder is emptied, the specimen saved. Patient is given 100 c.c. of water. The purpose of collecting two urine specimens is to prevent the possibility of incomplete emptying of the bladder.

As soon as the second specimen is obtained, blood is taken for urea determination. At 11 A.M. again empty the bladder and save the specimen. Emphasis must be laid on the fact that the patient must be kept quiet without any exercise during all this time.

Measure the volume of each specimen of urine, and calculate the amount per minute in cubic centimeters. Next, determine the urea concentration in each specimen; finally, determine the urea concentration in the specimen of blood withdrawn.

¹J. Clin. Invest. 6: 427, 1928.

Calculation.—

- (1) If the volume of urine excreted is more than 2 c.c. per minute, calculate the maximal clearance (C_m) by the following formula.

U = Urea concentration of urine.

B = Urea concentration of blood.

V = Volume of urine excreted per minute.

$$C_m = U \times V/B.$$

The average normal figure is 75, and the percentage of the normal = $1.33 \times U \times V/B$.

- (2) If the rate of excretion is less than 2 c.c. per minute, the standard clearance (C_s) is calculated according to the formula $C_s = U \times \sqrt{V/B}$. The average normal (with a urine volume of 1 c.c. per minute) is 54, and the percentage

$$\text{of normal} = \frac{1.85 \times U \times \sqrt{V}}{B}.$$

Values are expressed in % of the normal average maximum or standard clearance.

Example.—

- (1) (When V is greater than 2 c.c.)

Blood urea N is 12.2 mg. per 100 c.c. (B)

Urine urea N is 244 mg. per 100 c.c. (U)

Urine volume is 192 c.c. per hour, or 3.2 c.c. per minute (V)

Per cent of average normal maximum clearance is

$$1.33 \times 244 \times \frac{3.2}{12.2} = 85.12\%$$

- (2) (When V is less than 2 c.c.)

Blood urea N is 13.8 mg. per 100 c.c. (B)

Urine urea N is 750 mg. per 100 c.c. (U)

Urine volume is 48.1 c.c. per hour or 0.81 c.c. per minute (V)

$$\sqrt{0.81} = 0.9.$$

Per cent of average normal standard clearance is

$$\frac{1.85 \times 750 \times 0.9}{13.8} = 90.48\%$$

The following table from the Laboratory Methods of the U. S. Army, ed. 4, 1935, Lea and Febiger, is useful in obtaining the square root of V .

V c.c. per minute	\sqrt{V}	V c.c. per minute	\sqrt{V}
0.2	0.45	1.2	1.10
0.3	0.55	1.3	1.14
0.4	0.63	1.4	1.18
0.5	0.71	1.5	1.23
0.6	0.78	1.6	1.27
0.7	0.84	1.7	1.30
0.8	0.89	1.8	1.34
0.9	0.95	1.9	1.38
1.0	1.00	2.0	1.42
1.1	1.05	2.1	1.45

When the patient is a child, or an adult varying greatly from the average stature, a correction for size is required. Multiply the urine volume in these cases by the figure obtained from the formula $\frac{1.75}{\text{body surface in square meters}}$, and then use either equation (1) or (2) above.

URIC ACID

Uric Acid, Benedict, Using Folin Filtrate

Reagents.—

Stock Solution of Uric Acid (5 c.c. contain 1 mg. uric acid).—

Dissolve 9 gm. of pure crystalline hydrogen disodium phosphate (dibasic sodium phosphate, Na_2HPO_4) and 1 gram of dihydrogen sodium phosphate (monobasic sodium phosphate NaH_2PO_4) in 200 or 300 c.c. hot distilled water.

Filter and dilute to 500 c.c. with distilled water.

Pour this mixture, while still warm, on 200 mg. of pure dried uric acid (Kahlbaum) suspended in a few c.c. of distilled water in a liter volumetric flask.

Agitate until completely dissolved.

Add, at once, *exactly* 1.4 c.c. glacial acetic acid, c.p.

Dilute to 1000 c.c. with distilled water.

Add 5 c.c. of chloroform as a preservative.

This solution is not stable and must be made fresh each two months.

The uric acid should be dried overnight in a drying oven at 100°C . before weighing.

Stronger Uric Acid Standard, No. I (5 c.c. contain 0.05 mg. uric acid).

Place 25 c.c. of stock uric acid solution in a 500 c.c. volumetric flask.

Add 250 c.c. of distilled water and 25 c.c. of 10% solution of hydrochloric acid.

Dilute to 500 c.c. with distilled water.

This solution must be made fresh every two weeks.

Weaker Uric Acid Standard, No. II (5 c.c. contain 0.02 mg. uric acid).

Place 10 c.c. of stock uric acid solution in a 500 c.c. volumetric flask.

Add 250 c.c. distilled water and 25 c.c. of 10% hydrochloric acid.

Dilute to 500 c.c. with distilled water and mix.

This solution is stable for two weeks.

Uric Acid Reagent, Benedict. (Poison.)

Place 100 grams of sodium tungstate (Merck's Blue Label Brand) in a liter volumetric flask.

Add 600 c.c. distilled water, and dissolve.

Add 50 grams of pure arsenic acid and 25 c.c. of 85% phosphoric acid and 20 c.c. of concentrated hydrochloric acid.

Boil for about 20 minutes.

Cool and dilute to 1000 c.c. with distilled water.

This solution is stable.

5% Sodium Cyanide Solution.

5 gm. sodium cyanide in about 50 c.c. distilled water. Add 0.2 c.c. ammonium hydroxide and make up to 100 c.c.

(POISON. USE EXTREME CARE.)

Prepare every two months.

Equipment.—

- 4 5 c.c. volumetric pipettes.
- 3 large test tubes (1 × 8 inches).
- 1 1 c.c. pipette graduated in 0.1 c.c.
- 1 buret

Principle.—

The color produced by the action of the uric acid reagent with uric acid in protein-free blood filtrate is compared colorimetrically with the color produced by the same reagent with a standard solution of uric acid.

Technic.—

Use the Folin protein-free filtrate (page 224). Each c.c. contains 0.1 c.c. blood. Into a test tube marked "U" for unknown, place 5 c.c. Folin protein-free filtrate (containing 0.5 c.c. blood).

Add 5 c.c. distilled water.

In a test tube marked "S₁" for standard No. I, place 5 c.c. stronger uric acid standard, Folin (containing 0.05 mg. uric acid).

Add 5 c.c. distilled water.

In a test tube marked "S₂" for standard No. II, place 5 c.c. weaker uric acid standard, Folin (containing 0.02 mg. uric acid).

Add 5 c.c. distilled water.

To all three tubes, add from a buret 4 c.c. of 5% sodium cyanide.

Be sure the cyanide solution flows directly into the solution, and not down the sides of the tube, as a turbidity will result.

To each tube add 1 c.c. uric acid reagent, Benedict. (Poison.)

Mix by one inversion.

Work Rapidly

Place all three tubes immediately in a beaker of boiling water, and boil for three minutes.

All three tubes should be immersed in the water as nearly simultaneously as possible.

Remove and cool for three minutes in a beaker of cold water.

Read in the colorimeter, selecting the standard which most closely matches the color of the unknown.

Comparisons should be made within 5 minutes, or precipitates form.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 4 \text{ (if } S_2 \text{ is used) or } 10 \text{ (if } S_1 \text{ is used)} = \text{the number of}$$

mg. of uric acid in 100 c.c. blood.

Example.—

Reading of standard is 15; reading of unknown is 20; standard No. I was used.

$$\frac{15}{20} \times 10 = 7.5 \text{ mg. uric acid in 100 c.c. blood.}$$

Normal uric acid content is 2 to 3.5 mg. in 100 c.c. blood.

If Standard No. II were used, and the reading of the unknown were 20, the result would be $\frac{15}{20} \times 4$ or 3 mg. in 100 c.c. blood.

Improved Method for the Determination of Uric Acid in Blood, Folin¹**Reagents.—****Sodium Cyanide-Urea Reagent.—**

Transfer approximately 50 gm. of sodium cyanide to a 2 liter beaker. (Merck's best grade of coarsely granular sodium cyanide is recommended.)

Add 700 c.c. of distilled water and stir almost continuously until substantially complete solution is obtained.

Add 300 gm. of c.p. urea (Merck's), and stir. Complete solution is obtained in a few minutes, but the solution is not clear, usually because of impure urea.

Transfer the solution to a 2 liter flask.

Add a few gm. (5 or 6) of c.p. calcium oxide, and shake moderately for 4 or 5 minutes.

¹J. Biol. Chem. 86: 189, 1930.

The solution will now filter rapidly through a Whatman No. 41 filter paper. The filtrate will be free from carbonates and will yield 15 to 30% more color than before the removal of the carbonate. This solution if kept in a tightly stoppered bottle after filtration is stable for two months at room temperature and for many months if kept at refrigerator temperature. This cyanide solution when used with the uric acid reagent to be described has never (so far as we know) given a single turbid result.

Uric Acid Reagent.—

Transfer 100 gm. of sodium tungstate and 200 c.c. of distilled water to a 500 c.c. flask. Shake until the tungstate is dissolved.

Add slowly, with shaking and thorough cooling, 20 c.c. of 85% phosphoric acid.

Pass a rather slow hydrogen sulphide current through the solution for 20 minutes, and during the process (at the end of 3 or 4 minutes) add another 10 c.c. of 85% phosphoric acid. (See Chapter XV, Toxicology, for directions for generating H_2S .)

Filter through Whatman No. 41 filter paper.

Collect the first 40 c.c. of filtrate separately and pour back on the filter.

Transfer the filtrate to a separatory funnel (capacity 1 liter) and shake for a few minutes with 300 c.c. (1.5 volumes) of alcohol. The mixture separates into two layers.

Transfer the lower layer immediately into a previously weighed 500 c.c. flask. Discard the upper layer.

Add water to the mixture in the weighed flask until the weight of the contents reaches 300 gm.

Boil this solution for a few minutes to remove the H_2S .

Remove the flame and add 20 c.c. of 85% phosphoric acid. Boil slowly but continuously for one hour, using a condenser to prevent concentration. At the end of an hour, remove the flame, and decolorize with a few drops of bromine. Boil off excess bromine, and cool.

Transfer 12 gm. of lithium carbonate to a 500 c.c. beaker.

Add first 25 c.c. of 85% phosphoric acid and then slowly 50 c.c. of distilled water.

Boil to remove the CO_2 and see that the phosphoric acid has dissolved all of the carbonate.

Cool the lithium phosphate, mix with the concentrated uric acid reagent, and dilute to 1 liter.

Keep in well-stoppered bottles protected from light. This solution will retain its original slightly yellow color for months if kept away from dust and light, but only a day or two of strong sunlight will produce some blue color. The solution in daily use should, therefore, be kept in brown bottles.

Stock Uric Acid Solution.—

Weigh on a watch glass exactly 1 gm. of uric acid and transfer it to a small funnel inserted in a liter volumetric flask. Tap the funnel, so as to transfer nearly the whole of the uric acid to the flask.

Transfer 0.6 gm. of lithium carbonate to a 250 c.c. Florence flask.

Add 150 c.c. of distilled water.

Shake until solution is obtained (5 minutes). Since some insoluble material remains, it is usually best to filter.

Heat the solution or filtrate to 60° C. Also warm the liter flask under running warm water. Pour the warm lithium carbonate solution into the flask, incidentally washing into the flask traces of uric acid which adhere to the watch glass and funnel. Shake so as to dissolve uric acid promptly. (A little additional warming under hot tap water is permissible.) The lithium carbonate solution is not always perfectly clear, even when filtered. Do not mistake this slight turbidity for undissolved uric acid and keep warming and shaking too long. In 5 minutes all the uric acid should be dissolved.

Shake the flask under running cold water without undue delay.

Add 20 c.c. of 40% formalin, and half fill the flask with distilled water.

Add a few drops of methyl orange solution and finally add 25 c.c. of normal sulphuric acid from a pipette, rather slowly and with shaking. The solution should turn pink, while 2 or 3 c.c. of the acid are still left in the pipette, thus showing that the total acidity from adding 25 c.c. of the acid is not great.

Dilute to volume, mix thoroughly, and transfer to a clean, tightly stoppered bottle. This stock solution, containing 1 mg. of uric acid in 1 c.c., should be kept away from the light.

Standard Uric Acid Solution (1 c.c. contains 0.004 mg. of uric acid).—

To prepare the working standard, dilute 1 c.c. of the stock solution with distilled water to 250 c.c. It behaves exactly like a lithium carbonate solution of uric acid. This solution should be prepared just before use. It is not stable.

Equipment.—

- 3 large test tubes (1 × 8 inches) graduated at 25 c.c.
- 1 3 c.c. volumetric pipette.
- 1 2 c.c. volumetric pipette.
- 2 5 c.c. volumetric pipettes.
- 1 1 c.c. pipette graduated in 0.1 c.c.

Technic.—

To a large test tube labeled "U" for unknown, add 5 c.c. of Folin protein-free filtrate (containing 0.5 c.c. blood). (See page 224.)

To a similar tube labeled "S₁," add 3 c.c. standard uric acid solution.

To a third tube labeled "S₂," add 5 c.c. standard uric acid solution.

To the tube marked "S₁," add 2 c.c. distilled water.

From a buret add 5 c.c. of cyanide-urea solution to each tube.

To each tube add 1 c.c. of uric acid reagent.

Mix and allow to stand 4 minutes. Heat in boiling water 2 minutes.

Cool and dilute each tube to 25 c.c. Mix and read colorimetrically.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times (\text{multiplying factor}) = \text{mg. of uric acid in 100 c.c. of blood.}$$

Note.—

If Standard 1 is used the multiplying factor is 2.4.

If Standard 2 is used the multiplying factor is 4.

Example.—

Reading of standard is 20; reading of unknown is 30; "S₂" was used.

$$\frac{20}{30} \times 4 = 2.67 \text{ mg. uric acid in 100 c.c. blood.}$$

Normal is 2 to 3.5 mg. uric acid in 100 c.c. blood.

LaMotte Blood Uric Acid Outfit

The LaMotte blood uric acid outfit utilizes a method of determining uric acid content of blood according to Folin, using the Folin-Wu filtrate. A portion of the blood filtrate is treated directly with the Folin uric acid reagent and sodium cyanide, and the blue color that is produced is compared with prepared color standards representing uric acid in milligrams per 100 c.c. of blood.

Uric Acid and Gout

Blood chemical investigations of the amount of uric acid in blood are useful, first, in determination of the beginning of interference with kidney function, and second, in including or excluding gout from the diagnostic chart. Gout was first absolutely described by Sydenham in 1683. At this time we

believe that it is a chronic disturbance of metabolism in which there is a noted accumulation of uric acid in the blood as a result of the disturbance of the exogenous and endogenous uric acid formation.

Garrod introduced the "Thread Test" in 1844 and wrote an important treatise on the subject in 1859. He noted that in gout there is excessive uric acid in the blood due to increased formation and due to decrease in elimination of uric acid. Although he had no scientific methods to prove his point, present-day methods completely corroborate this statement. Garrod also claimed that uric acid was increased in certain cases of nephritis, a fact which has also been confirmed.

The normal values given for blood uric acid are between 2 and 3.5 mg. in 100 c.c. of blood. These figures are altered by an increased formation or a decreased elimination, or an increase in the normal amount or speed of uric acid destruction in the blood. The principal increases of uric acid are due to the increased kidney function, although in gout we believe that the increase in the blood is due to the uric acid destruction in the blood. In gout one may find uric acid values as high as 10 mg. per 100 c.c. of blood. The uric acid test of blood is not an absolute proof of gout, but it is important in the differential diagnosis between gouty and nongouty arthritis. In all cases of gout the uric acid is increased and in many of the cases which simulate it, it is not increased.

In arthritis the amount of uric acid is not increased on a permanent basis. Occasionally, uric acid is increased in a nongouty arthritis, but this is only a temporary finding. In lead poisoning there is sometimes a rise in the uric acid content.

It is in nephritis that an increase in uric acid is expected in the beginning of certain types of cases—the chronic variety. The retention of uric acid, which is an observation in the early stages of chronic and acute nephritis, sometimes falls, due to dietary restrictions, with the progress of the disease, but when the urea nitrogen becomes increased, there is a coincident increase of the uric acid.

We have seen a number of cases of complications in nephritis with an increase in uric acid, such as intestinal obstruction, pneumonia, diabetes, and metallic poisonings. There is a very unusual increase in blood uric acid in eclampsia. An explanation of this has not been given. It does not seem to be associated with a degree of kidney defect which one might expect would cause it.

Uric acid is increased in leukemia, and in certain types of chronic eczema. Administration of drugs, such as salicylates, is accompanied by an increased excretion of uric acid in the urine, due to increased renal function. Since salicylates are largely employed in infectious arthritis, particularly because of their analgesic properties, it is noteworthy that they increase the elimination of uric acid. We believe, however, that the benefit derived from them is not dependent upon the excretion of the uric acid from the blood stream, but upon the analgesic qualities.

CREATININE

Creatinine (Preformed), Folin and Wu

Reagents.—

Stock Solution of Creatinine. (1 c.c. contains 1 mg. creatinine.)

Dissolve 1 gram of creatinine, c. p., in 1000 c.c. N/10 hydrochloric acid in a liter volumetric flask.

This must be mixed thoroughly to insure solution. It is well to dissolve the creatinine in a small quantity of the hydrochloric acid in the volumetric flask, then dilute to volume with the N/10 hydrochloric acid.

Standard Creatinine Solution. (1 c.c. contains 0.006 mg. creatinine.)

Add 6 c.c. stock creatinine solution (1 c.c. contains 1 mg. creatinine) to 10 c.c. N/10 hydrochloric acid in a liter volumetric flask.

Mix, and dilute to 1000 c.c. with distilled water.

Add 5 drops of toluene as a preservative.

Alkaline Picrate Solution.

This solution is prepared fresh for each test. It does not keep.

To 50 c.c. saturated solution picric acid in a small beaker,

add 10 c.c. of freshly prepared 10% sodium hydroxide solution and mix.

Equipment.—

- 5 large test tubes.
- 1 100 c.c. lipless cylinder.
- 4 10 c.c. volumetric pipettes.
- 3 5 c.c. volumetric pipettes.
- 2 15 c.c. volumetric pipettes.
- 1 20 c.c. volumetric pipette.

Principle.—

Protein-free blood filtrate and a standard solution of creatinine are treated with an alkaline picrate solution. Picric acid is reduced to picramic acid and the red color produced by the latter in an alkaline solution is compared colorimetrically with the standard solution. Comparison should be made within 15 minutes.

Technic.—

Use 5 large test tubes, marked respectively, "U" for unknown, "S₁" for standard No. I, "S₂" for standard No. II, "S₃" for standard No. III, and "S₄" for standard No. IV.

Make alkaline picrate solution by adding

to 50 c.c. saturated picric acid

10 c.c. 10% sodium hydroxide.

Into tube "U," place 10 c.c. Folin filtrate (containing 1 c.c. blood).

In tube "S₁," place 5 c.c. standard creatinine solution, Folin (1 c.c. contains 0.006 mg. creatinine).

In tube "S₂," place 10 c.c. standard creatinine solution.

In tube "S₃," place 15 c.c. standard creatinine solution.

In tube "S₄," place 20 c.c. standard creatinine solution.

Add 15 c.c. distilled water to tube "S₁."

Add 10 c.c. distilled water to tube "S₂."

Add 5 c.c. distilled water to tube "S₃."

No water is added to tube "S₄."

Mix the contents of each tube.

To tube "U," add 5 c.c. alkaline picrate solution.

To each of the standard tubes, add 10 c.c. alkaline picrate solution.

Mix, and let stand eight to ten minutes.

Select the standard which most closely matches in color the color of the unknown.

Read in the colorimeter.

Calculation.—

$$\frac{\text{Reading of the Standard}}{\text{Reading of the Unknown}} \times \text{Factor for standard} = \text{the number of mg. of creatinine in 100 c.c. blood.}$$

Factor for standard: for "S₁," 1.5; for "S₂," 3.0; for "S₃," 4.5; for "S₄," 6.0.

Example.—

Reading of standard, 15; reading of unknown, 10; standard No. 1 used.

$$\frac{15}{10} \times 1.5 = 2.25 \text{ mg. creatinine in 100 c.c. blood.}$$

Normal is 1 to 2 mg. in 100 c.c. blood.

LaMotte Blood Creatinine Outfit

This outfit utilizes the method of determination of preformed creatinine, according to the method of Folin, using the Folin-Wu blood filtrate. A portion of the protein-free filtrate is treated with an alkaline picrate reagent and the resulting color then compared with accurately standardized creatinine standards contained in the set. These standards cover a wide range, so that with nephritic bloods of abnormal creatinine values a standard is always available.

CREATINE**Creatine, Folin****Reagents.—**

The reagents for this test are the same as used in the test for Creatinine, Folin Method, above.

N/1 hydrochloric acid is required in this test. See page 24 for normal solutions.

Equipment.—

- 1 100 c.c. graduated cylinder.
- 3 10 c.c. volumetric pipettes.
- 1 25 c.c. volumetric flask.
- 2 5 c.c. volumetric pipettes.
- 1 1 c.c. pipette graduated in 0.1 c.c.
- 1 50 c.c. volumetric flask.
- 1 2 c.c. volumetric pipette.

Principle.—

Folin blood filtrate is treated with an alkaline picrate solution and is compared colorimetrically with a standard solution similarly treated.

Technic.—

Make alkaline picrate solution. (See page 271.)

In a 25 c.c. volumetric flask, marked "U" for unknown, place 5 c.c. Folin filtrate (containing 0.5 c.c. blood) (page 224) and 1 c.c. N/1 hydrochloric acid.

Cover with tin foil and autoclave at 20 pounds pressure for twenty minutes. Cool. In a 50 c.c. volumetric flask, marked "S" for standard, place 10 c.c. standard creatinine solution, Folin (1 c.c. contains 0.006 mg. creatinine).

Add 2 c.c. N/1 hydrochloric acid.

To the unknown ("U") add 5 c.c. alkaline picrate solution.

To the standard ("S") add 10 c.c. alkaline picrate solution.

Allow both to stand eight to ten minutes.

Dilute the unknown to 25 c.c. with distilled water, and the standard to 50 c.c. with distilled water.

Read in a colorimeter.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 6 = \text{mg. creatine plus creatinine in 100 c.c. blood.}$$

Subtract the creatinine from the total and multiply by 1.16 to obtain the amount of creatine.

Example.—

Reading of standard, 15; reading of unknown, 10;

$$\frac{15}{10} \times 6 = 9 \text{ mg. of creatinine and creatine in 100 c.c. blood.}$$

There were 2.25 mg. of creatinine in the blood.

Creatine content is $9 - 2.25$, or $6.75 \times 1.16 = 7.83$ mg. in 100 c.c. blood.

Normal is 5 to 6 mg. in 100 c.c. blood.

TOTAL NITROGEN IN BLOOD**Total Nitrogen, Micro-Kjeldahl, Modified*****Reagents.—**

50% Sulphuric Acid.—

30% Hydrogen Peroxide.—

Keep in a refrigerator.

Stock Ammonium Sulphate Solution.—

Dissolve 9.4332 gm. of ammonium sulphate, reagent grade, in N/5 sulphuric acid in a liter volumetric flask, and dilute to 1,000 c.c. with N/5 sulphuric acid. 1 c.c. contains 2 mg. nitrogen.

Standard Nitrogen Solution.—

Add 20 c.c. stock ammonium sulphate solution (above) to 180 c.c. N/5 sulphuric acid in a liter volumetric flask.

Dilute to 1,000 c.c. with distilled water.

1 c.c. contains 0.04 mg. nitrogen.

This solution is stable.

N/5 Sulphuric Acid.—

Dilute 5.6 c.c. of concentrated sulphuric acid, sp. gr. 1.84, to 1,000 c.c. with distilled water in a liter volumetric flask.

Nessler's Reagent.—

See page 244.

Technic.—

Place 1 c.c. of blood in a 200 c.c. volumetric flask.

Dilute to 200 c.c. with distilled water.

Mix.

When hemolysis is complete, place 1 c.c. of diluted blood in a test tube, 1 by 8 inches, graduated at 35 and 50 c.c.

Add 1 c.c. of 50% sulphuric acid.

Heat carefully with a microburner, keeping the tube in a vertical position. Continue heating until dense white fumes fill the tube. Carry out this procedure under a hood.

Cool one-half minute and add 2 drops of 30% hydrogen peroxide directly to the solution.

Heat again until the white fumes appear.

If the brown color fails to disappear, repeat with 30% hydrogen peroxide.

Boil for one minute.

Cool and dilute to 35 c.c. with distilled water.

Into a similar tube marked "S" for standard place 5 c.c. of standard nitrogen solution and dilute to 35 c.c. with distilled water.

Place both tubes in a water bath at 25° C.

Add 15 c.c. of Nessler's reagent to each tube.

Mix and return to the water bath.

After 5 to 20 minutes compare in a colorimeter.

Calculation.—

$$\frac{\text{Reading of the Standard}}{\text{Reading of the Unknown}} \times 40 = \text{mg. nitrogen per c.c.}$$

*See method on page 253.

Example.—

Reading of standard was 15; reading of unknown was 18.

$$\frac{15}{18} \times 40 = 33 \text{ mg. nitrogen per c.c. of blood.}$$

$$33 \times 100 = 3300 \text{ mg. per 100 c.c. of blood, or 3.3 grams, or 3.3\% nitrogen.}$$

Normal.—3 to 3.7% nitrogen.

Kjeldahl Method**Reagents.—**

Concentrated Sulphuric Acid, C.P.

Copper Sulphate, C.P.

Ammonia-Free Water.

Saturated Solution of Sodium Hydroxide.

Few Pieces of Granulated Zinc.

Small Piece of Paraffin, or use Caprillic Alcohol for Antifoaming Solution.

N/10 Sulphuric Acid.

This may be prepared from a stock N/1 sulphuric acid by a 1 to 10 dilution.

Congo Red Indicator.—

To 90 c.c. of distilled water add 10 c.c. of 95% alcohol.

Add 0.5 gm. of Congo red, and dissolve thoroughly.

N/10 Sodium Hydroxide.

Dissolve 5 gm. of sodium hydroxide (Merck Reagent) in 1000 c.c. of distilled water.

Add 2.5 c.c. of 25% barium chloride (aqueous) solution.

Mix thoroughly and filter through dry filter paper.

Use phenolphthalein (0.5% alcoholic solution) as an indicator.

Titrate this against a N/10 oxalic acid prepared by dissolving 6.303 gm. of oxalic acid (Merck Reagent) in 500 c.c. of distilled water and diluting to 1000 c.c. with distilled water.

The N/10 sodium hydroxide was purposely made too strong.

Therefore, less than 50 c.c. of sodium hydroxide will be required to neutralize 50 c.c. of N/10 oxalic acid.

Suppose that 48 c.c. of sodium hydroxide were required to neutralize 50 c.c. of oxalic acid, and that upon measuring the sodium hydroxide solution there were 900 c.c.

$48 : 50 :: 900 : x$. x = total volume to which the sodium hydroxide must be diluted.

$$48x = 900 \text{ times } 50; \text{ or } 48x = 45000.$$

$$x = 937.5 \text{ c.c.}$$

Dilute the sodium hydroxide to 937.5 c.c.

Caution.—Do not let sodium hydroxide solutions stand exposed to the air, as the sodium hydroxide content will become lessened. Make a final titration of the sodium hydroxide against the oxalic acid as a check over the dilution.

1 c.c. of N/10 sodium hydroxide should exactly neutralize 1 c.c. of N/10 oxalic acid.

Technic.—

In a long-necked Jena glass Kjeldahl flask place 1 c.c. of blood.

Add 20 c.c. of concentrated sulphuric acid and about 0.2 gm. of copper sulphate.

Boil the mixture in the digestion rack for some time after it is colorless (about one hour).

Allow the flask to cool.

Dilute the contents with 200 c.c. of ammonia-free water.

Add about 40 c.c. of saturated sodium hydroxide solution. (A little more than is necessary to neutralize the sulphuric acid.)

Introduce into the flask a little coarse pumice stone or a few pieces of granulated zinc to prevent bumping.

Add a small piece of paraffin or a few drops of caprylic alcohol to lessen the tendency to froth.

By means of a safety tube, connect the flask with a condenser. This is so arranged that the delivery tube passes into a vessel containing a known volume (the volume used depending upon the nitrogen content of the blood) of N/10 sulphuric acid to which has been added a few drops of Congo red indicator. Take care that the end of the delivery tube reaches beneath the surface of the fluid. The delivery tube should be of a large caliber in order to avoid the sucking back of the fluid.

Mix the contents of the distillation flask very thoroughly by shaking or rotating.

Distill the mixture until about 2/3 of the solution has passed over.

Titrate the partly neutralized N/10 sulphuric acid against a N/10 sodium hydroxide.

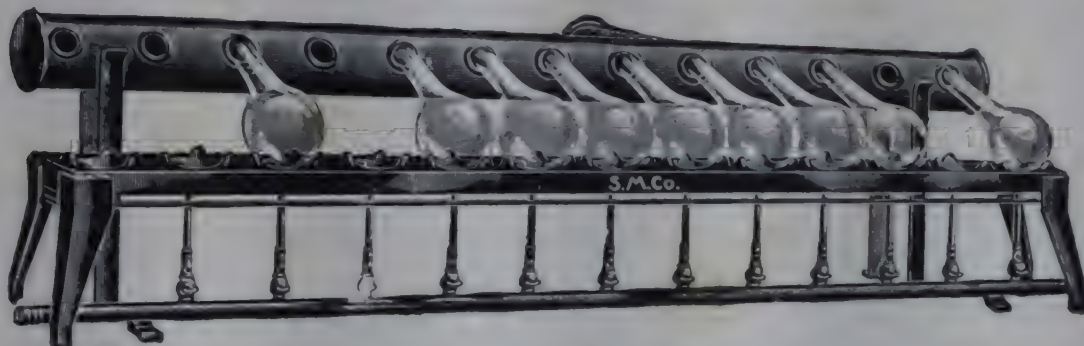


Fig. 105.—Digestion rack. (Gradwohl and Blaivas.)

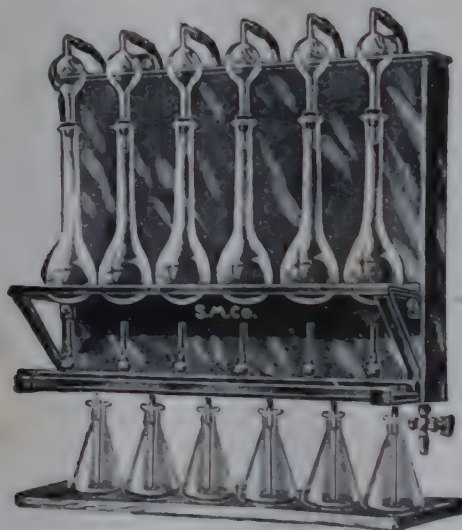


Fig. 106.—Kjeldahl apparatus with condenser. (Gradwohl and Blaivas.)

Calculation.—

1 c.c. of N/10 sulphuric acid is the equivalent of 0.0014 gm. of nitrogen.

Subtract the number of c.c. of N/10 sodium hydroxide used in the titration from the number of c.c. of N/10 sulphuric acid that were used in the test.

Multiply the remainder by 0.0014 = the grams of nitrogen present in 1 c.c. of blood.

Multiply this result by 100 = the nitrogen present in 100 c.c. of blood.

Normal Total Nitrogen.—3.3.7%.

INTERPRETATION OF BLOOD CHANGES IN NEPHRITIS

(See Section on Nephritis, pages 181 ff.)

The tests which have just been catalogued are of inestimable value in the determination of kidney function. Their use, together with the phenolsulphonephthalein test of Geraghty and Rowntree, have done a great deal to

advance the exact diagnosis of kidney disease. In connection with the phenolsulphonephthalein test, the blood chemical methods for determination of the quantity of nonprotein nitrogenous constituents have placed exact diagnosis or prognosis of renal disturbances in a very clear situation. The phenolsulphonephthalein test, while useful in determining the function of the kidney for the moment, does not represent the condition of the kidneys so far as actual retention of objectionable constituents is concerned. The blood chemical tests, however, represent the exact condition of the kidney over a long period of time and are, therefore, to be preferred.

The fact that the phenolsulphonephthalein test and the blood findings do not go hand in hand was proved experimentally by Frothingham, Fitz, Folin, and Denis.¹ Rabbits suffering with nephritis experimentally induced by the injection of uranium nitrate were also tested by the Geraghty and Rowntree method. As a result of these experiments, it was found that the phenolsulphonephthalein test is a good test to show the condition of the kidney for the moment, but that the blood chemical tests are more important to give a better idea of the general state of functional activity of the kidneys.

The first accumulation in the blood when kidney function is interfered with by beginning chronic nephritis is in the uric acid values, next there occurs an accumulation of urea as well as uric acid, and finally, in uremic nephritis we find an accumulation of uric acid, urea nitrogen, and creatinine. This seems particularly interesting and important in view of the fact that the urinary changes in some of these cases are exceedingly scant. Albumin and casts are often found, but this gives the clinician little information with regard to the true metabolic processes that are going on and the exact state of kidney function. We cannot well understand how a clinician can safely pass judgment in a case of chronic nephritis without an examination of the blood for these ingredients.

We know that the greatest amount of retention of urea, uric acid, and creatinine occurs in chronic nephritis particularly when uremia is present. A prognostic sign of no mean importance is that first pointed out by Myers and Lough.* They showed at that time (1915) that when creatinine in the blood appeared in the concentration of 5 mg. per 100 c.c. of blood and over, every one of these cases terminated fatally. Of the eleven cases in their series showing over 5 mg. of creatinine per 100 c.c. of blood, all terminated fatally in from a few days to two months. In this group of cases the phenolsulphonephthalein output was practically zero, with but one exception. These cases of creatinine values of 5 mg. or above were: a case of mercuric bichloride poisoning, with creatinine value of 33.3 mg.; a case of chronic nephritis in uremia with creatinine of 20.5 mg.; six other cases of nephritis, with creatinine values of 20.0, 16.7, 16.6, 14.7, 11.0 and 5.3 mg., respectively; three cases of chronic nephritis, with uremia, with creatinine values of 14.7, 7.4, and 7.0 mg. of creatinine, respectively. They had, at that time, three times as many cases on record in which this fact was borne out.

*Myers and Lough: Arch. Int. Med. 10: 536, 1915.

¹Frothingham, Fitz, Folin, and Denis: Arch. Int. Med. 12: 145, 1913.

The prognostic value of the finding of 5 mg. of creatinine or over as indicative of a fatal outcome is not infallible; we have seen several cases with over 5 mg. of creatinine per 100 cc. of blood that did not end fatally. We are able, however, to state that in many cases, it is a very significant finding. In a series of cases of thermic fever, studied at the St. Louis City Hospital by Gradwohl and Schisler,¹ we found some fatal cases with a high creatinine content although their other clinical symptoms did not suggest a fatal outcome. All patients with this high creatinine content died within from twenty-four to forty-eight hours.

Hyperthyroidism and Blood Sugar Changes

(See Protein-Bound Iodine, pages 478 ff.)

Cases of hyperthyroidism frequently show high blood sugar, although not often while the patient is fasting, and alimentary glycosuria may be easily induced in this condition, as has been pointed out by Hamman and Hirschman, 1917; Denis, Aub, and Minot, 1917; Janney and Isaacson, 1918.

Blood sugar observations obtained after glucose tolerance tests give much more definite information regarding the carbohydrate tolerance of individuals with endocrine disorders than studies made on the urine. Experimental proof that hyperglycemia results from hypoendocrine function was obtained by Janney and Isaacson; hypoglycemia regularly developed after thyroidectomy. Low blood sugar values, from 0.06 to 0.09 per cent, have been reported in myxedema, cretinism, Addison's disease, pituitary disease, and other less clearly defined conditions such as muscular dystrophy. In pronounced hypoendocrine conditions there is very little rise, if any, in the blood sugar after ingestion of glucose (1.75 grams per kilo of body weight) employed for the carbohydrate tolerance test, but in hyperthyroidism the blood sugar one hour after taking the glucose generally shows 0.2 per cent or more in contrast to the normal rise of 0.15 per cent.

Blood Chemistry and Surgery

Operative risk is largely judged by kidney function. Operative risk means ability to stand the anesthetic and to carry on the function in the presence of an overwhelming change in the organism caused by the operative attack. The methods formerly in vogue in surgical institutions to judge kidney function were the routine urinalysis and the phenolsulphonephthalein test for kidney efficiency. From what has gone before, it seems rational to include in this survey of the patient a very complete blood chemical analysis. Since the data already obtained by blood chemical methods have so often upset and changed medical diagnoses, and prognoses, it goes without saying that the same set of conditions will occur when these tests are used in connection with surgical procedures. Certainly the surgeon who proceeds to operate after having been assured that the blood sugar, urea nitrogen, uric acid, and creatinine of his patient are within normal bounds will have far less cause for fear of unforeseen catastrophe to his patients than those who rely simply on the tests commonly used with respect to the urine. Possibly in no department of surgery are these tests so much indi-

¹Gradwohl, R. B. H., and Schisler, E.: *Am. J. M. Sc.* 154: 407, 1917.

cated as in urology in connection with operative procedures upon the old men candidates for prostatectomy. Remarkable lowering of the death rate from this operation has occurred since the institution of rational preparation of these bad risks for surgery has been carried out, with free washing of the kidneys for days prior to the operation by copious drinking of water, the use of diuretics, the awaiting until cardiac and renal functions are within normal limits. These patients formerly were examined by the routine methods of urine analysis, special attention being paid to the output of urea without much attention to the blood findings. Estimation of urea without blood urea determinations is necessarily of but little scientific benefit. These tests should be supplemented by blood urea estimations and nonprotein nitrogen as well as blood sugar and uric acid and creatinine tests.

Aside from the preliminary survey of these operative patients, the surgeon may well utilize the methods of blood chemistry for determination of the impending onset of acidosis in his patients after operation. We hear much of the term "acidosis" in the surgical hospital, but hear but little of its exact diagnosis. Certain it is, much that is called acidosis in the course of a surgical operation is not acidosis at all and perhaps cases of acidosis occur that are never recognized. It is here that blood chemistry must come forward to settle this question. A rapid estimation of the CO_2 combining power of the patient's blood plasma by the Van Slyke or Marriott method will speedily clear the picture so far as acidosis is concerned.

In connection with the use of blood chemical methods in surgery, Gradwohl and Scherck¹ reported before the American Urological Association at the 1917 meeting results with these methods in estimating kidney function in surgical cases, confining their work mainly to obstructive conditions of the lower urinary tract in which there was more or less back pressure on the kidneys. Some of these cases suffered from nephritis as well. In this paper we stated our views on this question about as follows: "these investigations showed that there was a relationship between an increase in the nonprotein nitrogenous constituents and the phenolsulphonephthalein. In all cases, the blood chemical analyses showed the true condition of the patients no matter what the clinical signs or urinary examinations indicated. In some cases, the phenolsulphonephthalein output was improved, although the patient became worse. In some cases where the blood chemical findings were normal, the phenolsulphonephthalein elimination was decreased. In one case the phenolsulphonephthalein output was disregarded in surveying operative risk, the patient was operated, relying in each case on the blood chemical findings, convalescence was in no manner unusual or disturbed by any thought of kidney insufficiency, such as was indicated by the diminished phenolsulphonephthalein output. In some cases there were extensive changes in the kidney without change in the phenolsulphonephthalein output, and yet with very definite retention of urea, uric acid, and creatinine. In general, it can be stated that the estimation of kidney function in connection with surgical operations

¹Gradwohl and Scherck: *Interstate M. J.* 24: No. 9, 1917.

is a matter of computation of a number of factors, and that the phenolsulphonephthalein test occupies a subordinate position, even when positive, and then it is of much more importance than when negative."

In general it may be stated that patients with urea nitrogen values over 30 mg. or with a phthalein excretion of under 40 per cent are to be regarded as dubious surgical risks.

The significance of the urea clearance of a given patient is important in estimating renal function. While it will not give us information about minor degrees of impairment, still it is generally considered that it gives the maximum information of renal function in nephritis of any single method. Urea clearance is not reduced in the earliest stages of hypertension or arteriosclerotic kidney disease. It has been found that nitrogen retention does not appear in these cases until the clearance is below 40 per cent and is not particularly marked until it is below 20 per cent. Uremic symptoms are noted when the clearance falls from 10 to 5 per cent. It is interesting, too, to know that the clearance figure rises as the patient recovers from an acute attack.

The surgeon must avail himself of as many laboratory examinations as are possible in handling preoperative and postoperative conditions. When supplemented by a series of studies concerning water electrolyte and nutritional imbalance, the surgeon is in an excellent position to evaluate his case. The weight loss by acute dehydration and lack of food intake is for the greatest part caused by the loss of body water. A gradual weight gain is associated with a satisfactory clinical picture and is a good criterion of rehydration.

Next, the complete blood counts, sedimentation rates, and hematocrit examinations aid in detecting hemoconcentration as the plasma volume is diminished in hypotonic dehydration. The presence or absence of anemia is of utmost importance because the oxygen-carrying capacity of the blood should be at an optimum point. With transfusion we can restore and correct anemia by increasing blood volume to normal limits. This improves the patient's tolerance for surgery. The sedimentation rate tells us of the behavior of plasma proteins. Urinalysis, a 6-hour urine volume, urine concentration tests, quantitative urine chloride, pH concentration every six hours all have value. Simple urinalysis will give one information regarding renal function, damage by overconcentration, presence of pus and red cells, casts, albumin, sugar. Six-hourly estimations of urine volume provide a closer watch upon the seriously ill patient.

It is well known that the body economizes sodium chloride much more efficiently than it does water, and with normal kidneys there is prompt cessation of the sodium chloride excretion in the urine in salt deficits. With normal kidneys and diminished or absent urinary chloride, one may assume that there is salt depletion and concentration of salt is going on in various undetermined degrees. With treatment one may see chlorides reappear in the urine as a positive sodium balance is attained.

Whenever possible, one should know the maximum urinary specific gravity in each patient. Diminished renal concentrating power is common and occurs in infancy, old age, obstructive uropathy, and as a result of any severe

illness, or following shock. It may persist for days. The minimum excretory volume of urine is that amount that is just sufficient for excretion of waste products. This volume depends on the renal concentrating power and amount of waste products presented for excretion.

We estimate blood urea nitrogen or nonprotein nitrogen to determine the presence or absence of retention of end products of protein metabolism or to detect intraluminal gastrointestinal bleeding.

In all these undertakings, serum sodium, potassium, and chloride must be estimated to evaluate the presence of extracellular deficit. It must be remembered that extracellular volumes and concentrations may not reflect deficits in the intracellular compartment because of compensatory mechanisms. If the values are low, an intracellular deficit certainly exists. Here come into play the various tests developed by flame photometry.

Other important information is to be obtained from nonprotein nitrogen- or plasma-protein determinations with albumin-globulin fraction. These figures are important in the aged.

The surgeon can determine by 24-hour urinary nitrogen tests a good idea of the clinical index of protein metabolism and thus aid in ascertaining the presence or absence of positive nitrogen balance.

In patients critically ill, the caloric fluid intake parenterally or by mouth should be recorded and evaluated each six hours or even more frequently. One should not forget that serum and urinary calcium and phosphate determinations may be helpful occasionally. In the presence of alkalotic tetany such measurements are confirmatory. Also serum cholesterol may be helpful in evaluating the presence of atherosclerosis and arteriosclerosis.

PROTEIN

Determination of Hemoglobin, Sheard-Sanford*

Reagent.—

Sodium Carbonate, 0.1%.—

Dissolve 1 gm. of anhydrous sodium carbonate in distilled water, in a liter volumetric flask.

Dilute to 1 liter with distilled water. Mix.

Technic.—

Place exactly 20 c.c. of 0.1% sodium carbonate in a 25 c.c. test tube.

Using a capillary pipette, calibrated "to contain," measure exactly 0.1 c.c. of well-mixed venous blood, or finger blood.

Blow the blood into the sodium carbonate solution, then carefully wash out the pipette by sucking up the sodium carbonate solution into the pipette and blowing it out. Repeat several times. The sodium carbonate solution prevents the formation of acid hematin; it serves, too, to prevent the trace of clouding that might occur from precipitation of globulin.

Stopper the tube and shake for 10 to 15 seconds.

Read in the photometer, using the green filter and setting the instrument to read 100 for distilled water. Little change will be observed in the reading for several hours.

If desired, 0.05 c.c. of blood may be used with 10 c.c. of sodium carbonate solution.

Calculation.—

A chart obtained by determining the oxygen capacity of fresh, normal blood by the Van Slyke manometric method using serial dilutions of blood, and calibrated for the instrument

*From Photoelectric Clinical Chemistry, by William S. Hoffman, 1941, William Morrow and Co., Inc., by permission.

and filter being used, is furnished with the photelometer at the time of purchase. Use this chart or curve for calculation.

If desired, standardization may be made by the iron method of Wong, below. The dilutions are made most easily by measuring 0.1 c.c. of the standardized blood sample into 16, 20, 25, 30, 40, and 50 c.c., respectively, of 0.1 N sodium carbonate solution. The values obtained with the green filter represent readings for blood samples containing, respectively, 1.25, 1.00, 0.80, 0.677, 0.50, and 0.40 times that of the original sample.

Thus, if the original sample showed an oxygen capacity of 20.3 c.c. per 100 c.c., which was equivalent to 15.15 gm. of hemoglobin per 100 c.c., then the readings obtained with the above dilutions were equivalent to 18.94, 15.15, 12.12, 10.10, 7.58, and 6.06 gm. per 100 c.c., respectively.

Plot these values on semi-logarithmic paper. They should form almost a straight line which goes through the 100 value at 0 concentration. (The error involved in not considering the color produced by the serum is too small to be determined.)

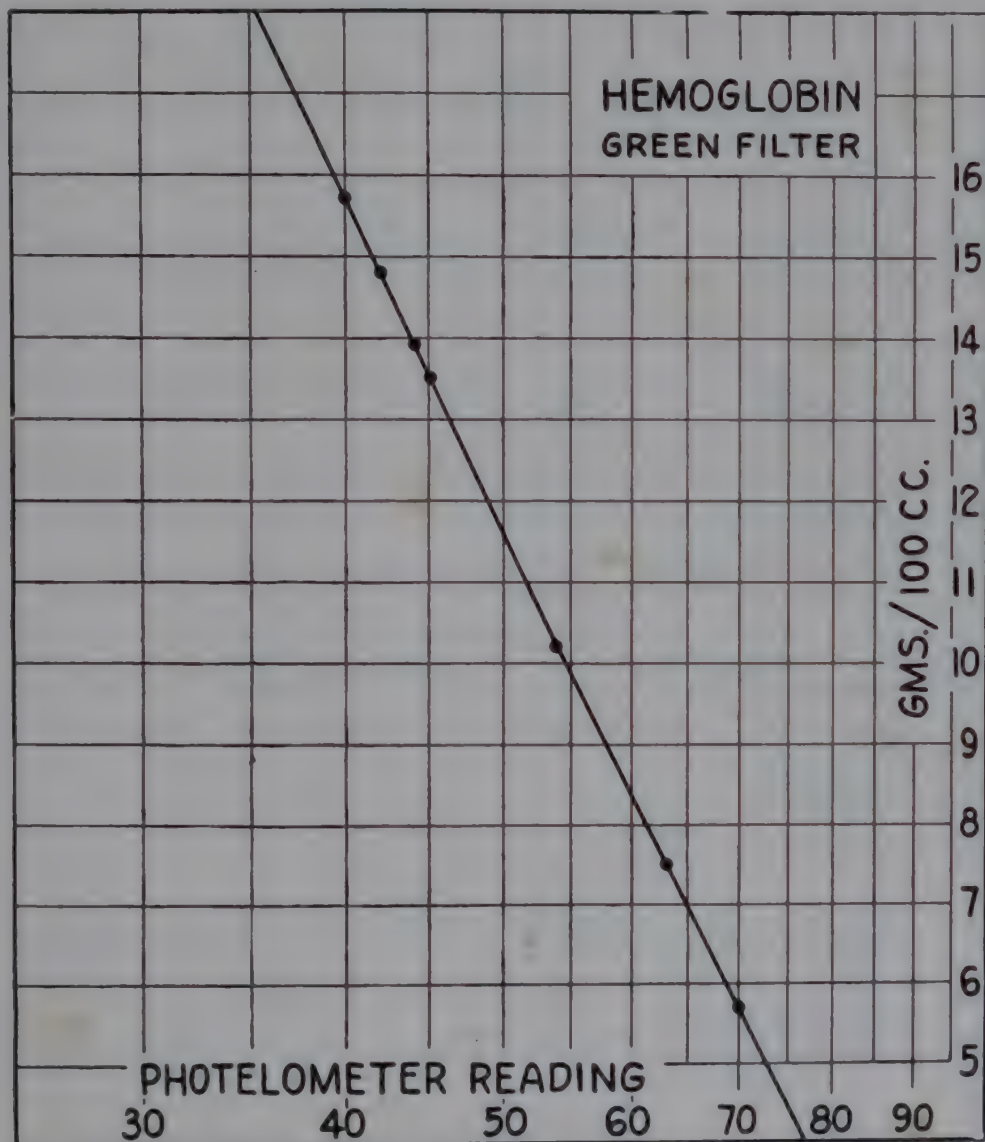


Fig. 107.—Typical calibration curve for hemoglobin. (Courtesy William S. Hoffman: Photometric Clinical Chemistry, William Morrow & Co., New York.)

Determination of Hemoglobin by Wong's Iron Method*

Reagents.—

Concentrated Sulphuric Acid, c.p.

Sodium Tungstate, 10%, as for Folin-Wu filtrates. (See page 225.)

Potassium Persulphate, saturated solution.

Transfer about 7 gm. of c.p. powdered potassium persulphate to a glass-stoppered 4 ounce bottle.

*From Photoelectric Clinical Chemistry, by William S. Hoffman, 1941, William Morrow and Co., Inc., by permission.

Add about 100 c.c. of distilled water, and shake until the solution is saturated. Allow excess solid to settle to the bottom. Use the supernatant liquid. This solution decomposes slowly but remains saturated with the solution of more of the excess solid.

Potassium Sulphocyanate, Approximately 3 N.—

Dissolve 29.2 gm. potassium sulphocyanate crystals, c.p. in distilled water in a 100 c.c. volumetric flask.

Add distilled water to about 90 c.c.

Add 4 c.c. acetone. Make up to 100 c.c. with distilled water. Filter if necessary.

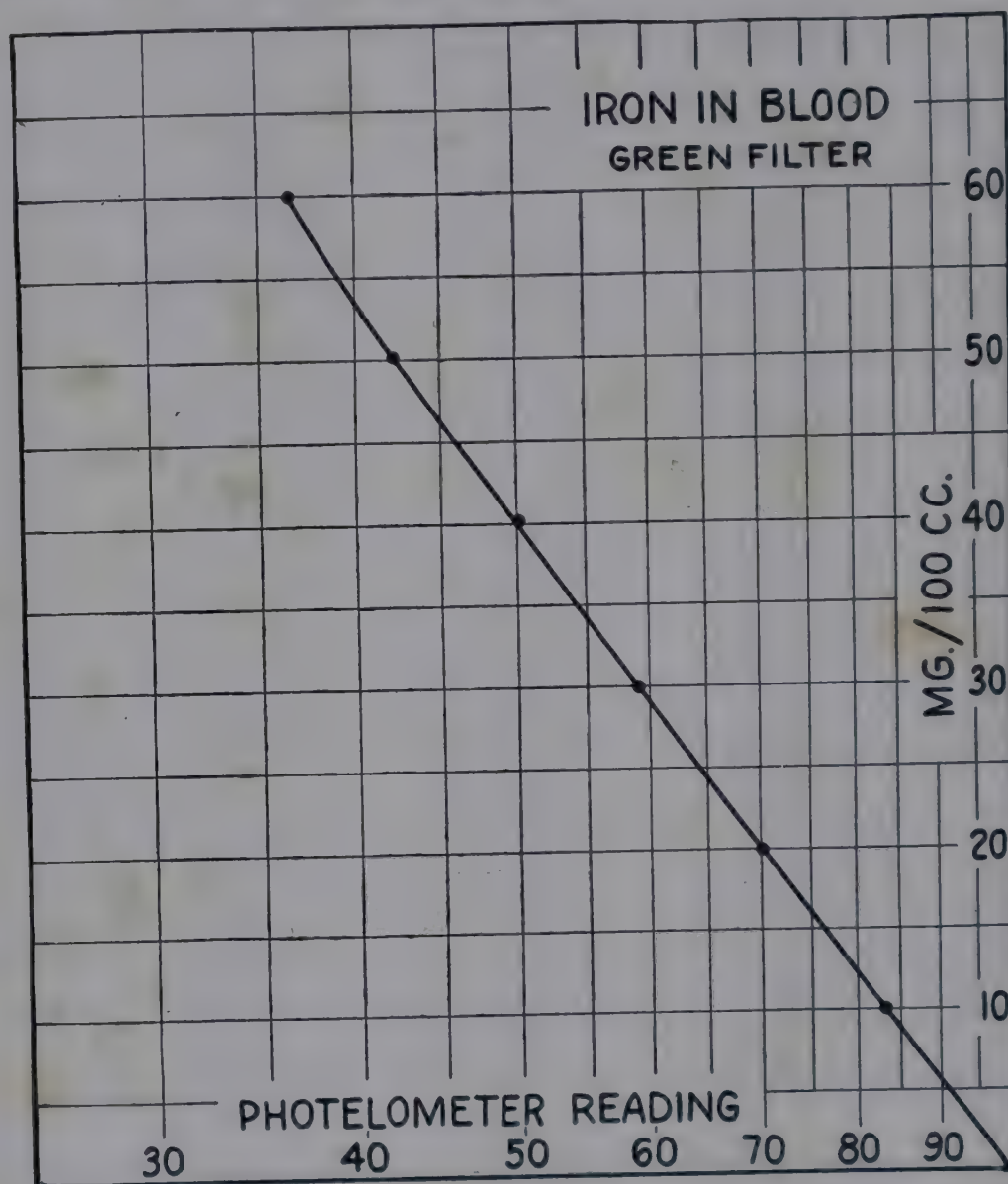


Fig. 108.—Typical calibration curve for total iron in blood. (Courtesy William S. Hoffman: Photelometric Clinical Chemistry, William Morrow & Co., New York.)

Technic.—

Transfer 0.5 c.c. of well-mixed blood, using an Ostwald-Folin pipette, to a 50 c.c. volumetric flask.

Add 2 c.c. of concentrated sulphuric acid.

Agitate the flask for several minutes.

Add 2 c.c. of saturated potassium persulphate solution.

Agitate the flask again.

Dilute carefully with about 25 c.c. of distilled water.

Cool to room temperature.

Add 2 c.c. of 10 per cent sodium tungstate solution.

Make up to the 50 c.c. mark with distilled water.

Mix for several minutes and filter, catching the first drops of filtrate in the original flask, and refiltering.

Transfer 15 c.c. of clear filtrate to a test tube graduated at 20 c.c.

Add 1 c.c. of saturated potassium persulphate solution and 3 c.c. of 3 N potassium sulphocyanate solution.

Dilute to 20 c.c. mark with distilled water, and mix by inversion, using a clean rubber stopper.

Read in the photelometer with the green filter, having set water at 100. The values do not change for at least an hour.

Calculation.—

Calculation is made from a calibration curve (see Fig. 107) prepared from a standard iron solution.

Standard Iron Solution.—

Dissolve exactly 0.8635 gm. of ferric ammonium sulphate crystals, c.p. ($\text{FeNH}_4(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$) in distilled water in a liter volumetric flask.

Add 2 c.c. concentrated sulphuric acid.

Cool. Dilute to 1 liter with distilled water.

Dilute Standard (100 c.c. contains 1 mg. Fe)

Place 10 c.c. of the standard iron solution in a 100 c.c. volumetric flask.

Dilute to 100 c.c. with distilled water.

Place 9.0, 7.5, 6.0, 3.0, and 0 c.c. of the dilute standard, respectively, into test tubes graduated at 20 c.c. These are equivalent to 15 c.c. of filtrates obtained from blood containing 60, 50, 40, 20, and 0 mg. Fe per 100 c.c., respectively.

To each solution add 0.6 c.c. concentrated sulphuric acid (or 3.6 c.c. of 6N H_2SO_4).

Cool to room temperature and

add to each, 1 c.c. of saturated solution of potassium persulphate solution and 3 c.c. of 3 N potassium sulphocyanate solution.

Dilute each to 20 c.c. with distilled water.

Stopper and mix.

Read in the photelometer, using the green filter. Plot the values on semi-logarithmic paper. They should fall on a slightly convex curve. (See Fig. 108.)

To 0.5 c.c. blood in a 50 c.c. volumetric flask

add 2.0 c.c. concentrated sulphuric acid. Agitate the flask.

Add 2.0 c.c. saturated potassium persulphate. Agitate, then dilute with about 25 c.c. distilled water. Cool.

Add 2.0 c.c. of 10% sodium tungstate.

Dilute to volume. Filter.

To 15 c.c. of filtrate in a test tube graduated at 20 c.c.

add 1 c.c. saturated potassium persulphate solution and 3 c.c. of 3 N potassium sulphocyanate.

Dilute to 20 c.c. Mix and read.

For methods of making determinations of other ingredients refer to "Photelometric Clinical Chemistry" by William S. Hoffman, published by William Morrow and Company, New York, 1941.

Hemoglobin in Blood

Modified Method of Sheard, Sanford, and Szigeti¹

Principle.—

The blood is laked in a solution of ammonium hydroxide and shaken with air. The concentration of oxyhemoglobin is then determined photometrically or spectrophotometrically.

Reagents.—

Ammonium Hydroxide, 0.04% Solution.—

Add 10 c.c. of reagent grade concentrated ammonium hydroxide to distilled water in a 250 c.c. volumetric flask and dilute to 250 c.c. with distilled water.

¹Am. J. Clin. Path. 23: 574, 1953.

For use: Dilute 10 c.c. of this stock solution to 1,000 c.c. with distilled water.

Green Filter, 545 m μ .—

Calibration Curve.—

Prepare in the usual manner of calibrating clinical hemoglobinometers (below).

Technic.—

Place 4 c.c. of 0.04% ammonium hydroxide in a test tube.

Add 0.02 c.c. of blood.

Rinse the pipette in the solution 3 times.

Stopper the tube and shake vigorously for 10 seconds.

Read in a photometer or spectrophotometer at a wave length of 545 m μ .

Calculation.—

Read the hemoglobin value directly from a calibration curve that has been prepared from the hemoglobin measurements by iron analysis.

Calibration of Clinical Hemoglobinometers.—

Place 14 to 15 c.c. of oxalated blood in a centrifuge tube and centrifuge at low speed for about 5 minutes.

Remove and discard about 2 c.c. of the supernatant plasma, using a pipette.

Thoroughly mix the remaining plasma with the red cells.

Dilute the blood serially:

Place 2 c.c. of blood in each of 6 test tubes numbered 1 through 6.

Add physiologic saline as follows:

Tube	1	2	3	4	5	6
	0.0	0.5	1.0	2.0	4.0	8.0 c.c.

Mix the contents of tube 1 thoroughly and immediately withdraw samples for the determination of iron and for the determination of hemoglobin by the clinical method of choice. It is best to prepare 3 determinations of each. Begin each determination (duplicates) by using the original specimen of blood.

Carry out the determinations on the remaining five diluted specimens in the same manner as for tube 1.

Calculate the hemoglobin equivalent for each of the prepared specimens of blood from the iron concentration.

Prepare a curve, plotting the average concentration of hemoglobin for each specimen of blood against the reading of that same specimen in the hemoglobinometer.

Copper Sulphate Method to Determine Specific Gravity of Blood and Hemoglobin Content*

This method makes it possible with three or four drops of blood, a medicine dropper, and small bottles of copper sulphate solution to determine the specific gravity of the blood, and from it the hemoglobin content within 10 per cent. The plasma protein concentration may also be determined. To measure the blood and plasma gravities and calculate the plasma proteins, hemoglobin, and hematocrit on the line charts requires about two minutes.

Principle.—

The technic consists of letting drops of plasma or whole blood fall into a graded series of solutions of copper sulphate of known specific gravity and noting whether the drops rise or fall in the solutions. Each drop on entering the solution becomes encased in a sack of copper-proteinate and remains as a discrete drop without change of gravity for fifteen or twenty seconds, during which its rise or fall reveals its gravity relative to that of the solution. The size of the drop does not have to be constant, hence no special pipette is needed. No temperature correction is required. The copper sulphate solution automatically cleans itself after each test, because within a minute or two after the test is completed the material of the drop settles to the bottom as a precipitate. The standard copper sulphate solutions are prepared by dilution of a stock solution of gravity 1.1000, which can either be prepared from

*Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Jr., Hamilton, P. B., and Archibald, R. M.: *Bull. U. S. Army Med. Dept.* 71: 66-83, December, 1943.

weighed amounts of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, or by dilution from a saturated solution of the sulphate. The latter procedure makes it possible to prepare the standard solutions without a balance.

For accurate work, a series of copper sulphate solutions graded at intervals of 0.001 in specific gravity is used; twenty solutions cover the plasma range 1.015-1.035 and forty cover the whole blood range, 1.035-1.075. For rougher work with gravities accurate to ± 0.001 , sixteen solutions at intervals of 0.004 suffice to cover the entire range of blood and plasma.

Apparatus.—

Apparatus for 'drawing and preparing blood:

Glass syringes, 5 or 10 c.c. capacity.

No. 20 hypodermic needles.

Rubber tourniquet.

Centrifuge (can be omitted if one can wait for blood to clot or sediment until a few drops of serum or plasma can be obtained).

Medicine droppers.

Oxalated test tubes, heavy-walled Pyrex, of about 10 c.c. capacity (125 by 16 mm.). These are prepared in advance for 5 c.c. portions of blood by pipetting into each tube 0.25 c.c. of a 2 per cent solution of 3:2 ammonium-potassium oxalate (see "Reagents"). The solution is spread in a film about the lower half of the tube and dried in an incubator (not over 50°C.) or in a vacuum desiccator. The tube is marked on the outside to hold 5 c.c.

Bottles or tubes for standard copper sulphate solutions: One or more of the following sets will be needed.

a. Laboratory set covering the ranges of blood, plasma, and transudates. Seventy oval or round bottles of 4-ounce capacity. This number will hold solutions covering the range 1.008-1.075 at 0.001 intervals and provide three spares. With these intervals the gravities can be estimated to ± 0.0002 . The 100 c.c. portions of solution held by these bottles suffice for analysis of about 100 blood specimens and their plasmas. The stoppers should be rubber, cork, or screw cap rather than glass, to prevent "freezing" and evaporation.

b. Portable set covering the same range. One-ounce bottles with screw caps. The 25 c.c. portions of solution held by these bottles suffice for analysis of about 25 blood specimens and their plasmas.

c. Field set for approximate analyses covering the range of blood and plasma. This includes sixteen bottles to hold solutions covering the range 1.016-1.074 at intervals of 0.004. It serves for gravities within ± 0.001 . The bottles may be 1-, 2-, or 4-ounce, according to the number of specimens of blood to be analyzed, and the space and weight desirable for the set.

d. Pocket field set for whole blood: This consists of six 1-ounce bottles to hold solutions of gravities covering the range 1.040-1.070 at intervals of 0.006. It serves to estimate blood gravities within ± 0.002 .

Apparatus for preparing the copper sulphate solutions: One volumetric flask of 100 c.c. capacity and one buret, preferably also of 100 c.c. capacity, for preparing standard copper sulphate solutions to be stored in 4-ounce bottles. If 2-ounce bottles are used, the flask and buret should be of 50 c.c. capacity; if 1-ounce bottles are used the flask and buret should be of 25 c.c. capacity.

Three 4-liter bottles.

One 1-liter volumetric flask.

One 500 c.c. graduated cylinder.

One 7-inch funnel, and cotton or filter paper.

One thermometer, Centigrade, for liquids at room temperature.

Reagents.—

2% Oxalate Mixture (3:2 Ammonium-Potassium Oxalate).—

Dissolve 3 gm. ammonium oxalate
and 2 gm. potassium oxalate
in 250 c.c. distilled water.

This oxalate mixture, suggested by Heller and Paul, disturbs cell and plasma gravities less than either potassium or ammonium oxalate alone.

Crystalline Copper Sulphate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.—

This is preferably purchased in the form of "fine crystals." Otherwise it must be pulverized before using. Four pounds provide a complete set (70) of 100 c.c. standard solutions. Ten pounds will probably suffice a laboratory a year.

Stock Copper Sulphate Solution of Gravity 1.1000.—**(a) By Weight.—**

If pure copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) can be weighed this procedure is simpler than the saturation method. Place in a dry 2-liter flask or bottle 170 grams of pure "fine crystals" of copper sulphate, weighed to within 0.1 gram.

Fill a 1-liter volumetric flask to the mark with distilled water, and add, according to the temperature of the water, from a graduated pipette to the liter in the flask, sufficient additional water to bring the volume up to that indicated in the second column of Table 14. Empty and drain the water from the flask into the bottle with the copper sulphate crystals. Stopper and rotate until all the crystals have dissolved. Then pour the solution once to and from the 1-liter flask, to mix the solution within the water left adherent in that flask.

Or place in a 500 c.c. flask or bottle 42.50 gm. of the copper sulphate, weighed within 0.02 gm.

Measure with a 250 c.c. volumetric flask and a graduated pipette the volume of water indicated in the last column of Table 14.

Dissolve and pour the solution back and forth as directed above.

For accuracy in this procedure, it is essential that the copper sulphate shall be pure and have the theoretical five molecules of water of crystallization. The crystals can be accurately analyzed by drying at 300° to 350° in Pyrex weighing bottles samples of 2 or 3 grams until the weight is constant, for which two or three hours suffice. The theoretical loss of weight for the change from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ to anhydrous CuSO_4 is 36.06 per cent. The analysis should be within 0.2 per cent of this. The analyzed copper sulphate must be kept in tightly closed bottles until it is used to make the solution, or it may lose some of the water of crystallization to the air. Even after the 170 or 42.5 gram portions of pure crystals have been weighed, they must be protected unless used at once; if they lost significant amounts of their crystal water the solution made according to Table 14 would have too little total water, and the gravity would be over 1.1000.

(b) From Saturated Copper Sulphate Solution.—**Stock Copper Sulphate Solution.—**

Prepare 4 liters of the stock solution. The following directions will yield that amount.

Saturation.—Provide three dry 4-liter bottles, a Centigrade thermometer for room temperatures, and an 8-inch funnel with a plug of cotton for rapid filtration. Fit the funnel to one end of the bottles.

In another 4-liter bottle place 4 pounds of "fine crystals" or pulverized crystalline copper sulphate and 2.5 liters of distilled water. (Tap water of gravity not over 1.0003, compared with distilled water at the same temperature as 1.0000, may be used.)

Stopper the bottle and shake vigorously, with repeated inversions, for five consecutive minutes by the watch. (It is convenient to have two operators who shake during the alternate minutes.)

The instant the shaking is finished take the temperature to the nearest half-degree. (It will be 2 or 3 degrees cooler than the water before the saturation because the saturation process absorbs heat.)

After taking the temperature, immediately decant the solution off the crystals through the funnel with the cotton plug filter, to filter out suspended small crystals.

Dilute the solution at once to make the stock solution of gravity 1.1000.

Two and one-half liters of the saturated solution suffice for more than 4 liters of the stock solution of gravity 1.100, and this in turn is sufficient for a complete set of 70 standard solutions of 100 c.c. volume each, with enough surplus to provide replacements for the standards which are most used. Smaller or larger amounts of the saturated solution can be made by using proportional amounts of copper sulphate and water.

TABLE 14.—VOLUME OF WATER TO ADD TO 170 OR 42.5 GRAMS OF $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ TO MAKE A STOCK SOLUTION OF GRAVITY 1.1000

TEMPERATURE OF WATER	C.C. OF WATER TO 170 GRAMS OF $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	C.C. OF WATER TO 42.50 GRAMS OF $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
38°	1000.2	250.0
36.	1001.2	250.3
34	1002.2	250.5
32	1003.2	250.8
30	1004.3	251.1
28	1005.3	251.3
26	1006.3	251.6
24	1007.4	251.8
22	1008.4	252.1
20	1009.5	252.4
18	1010.5	252.6
16	1011.5	252.9
14	1012.6	253.1
12	1013.6	253.4
10	1014.6	253.6

Dilution.—Measure the volume of saturated solution indicated in Table 15 into a 500 c.c. graduated cylinder and pour into a 1-liter volumetric flask.

Allow the upturned cylinder to drain into the flask for 30 seconds.

Fill the flask to the mark with water.

Invert several times to mix the solution. The mixing results in a contraction, so that the meniscus now falls below the mark.

Let the flask stand for a minute until the solution drains down from the neck.

Add enough additional water to bring the volume to 1 liter.

Mix and pour into a clean, dry, 4-liter bottle. Use the same 1-liter volumetric flask to prepare three more liters of the stock copper sulphate solution of gravity 1.100. Each time before the flask is used again, rinse it with water and discard the rinsings.

It is desirable that the saturated solution, the stock solution, and the standard solutions described below all be prepared at within 5° C. of the same temperature. The coefficients of expansion of the saturated and stock copper sulphate solutions are slightly greater than that of water, so that if, for example, the saturated and stock solutions

TABLE 15.—CUBIC CENTIMETERS OF SATURATED COPPER SULPHATE SOLUTION TO BE DILUTED TO 1-LITER TO GIVE THE STOCK SOLUTION OF SPECIFIC GRAVITY 1.100

Temperature in degrees C. refers to the temperature of the saturated solution at the time of saturation (end of shaking for five minutes)

TEMPERATURE		TEMPERATURE		TEMPERATURE	
C.°	C.C.	C.°	C.C.	C.°	C.C.
10.0	578	20.0	488	30.0	425
10.5	573	20.5	484	30.5	423
11.0	568	21.0	480	31.0	420
11.5	563	21.5	477	31.5	417
12.0	558	22.0	473	32.0	414
12.5	553	22.5	469	32.5	412
13.0	548	23.0	466	33.0	409
13.5	543	23.5	463	33.5	406
14.0	539	24.0	460	34.0	403
14.5	534	24.5	456	34.5	401
15.0	529	25.0	453	35.0	398
15.5	525	25.5	450	35.5	395
16.0	521	26.0	447	36.0	392
16.5	516	26.5	445	36.5	390
17.0	512	27.0	442	37.0	387
17.5	508	27.5	439	37.5	384
18.0	504	28.0	436	38.0	381
18.5	500	28.5	434	38.5	379
19.0	496	29.0	431	39.0	376
19.5	492	29.5	428	39.5	373
20.0	488	30.0	425	40.0	370

were prepared at 35° C. and the standard solutions at 20°, the standard solutions would have more copper sulphate than intended, enough to make their gravities about 0.001 too high.

Standard Copper Sulphate Solutions.—

In this method, four-ounce bottles are used to hold the standards.

Standard of 1.075 Gravity.—

Measure 74.3 c.c. of the stock solution of gravity 1.100 from a buret into a 100 c.c. volumetric flask.
Dilute to 100 c.c. with distilled water and mix.
Transfer to a labeled 4-ounce bottle, and stopper to prevent evaporation.

Standard of Gravity 1.074.—

Rinse the 100 c.c. volumetric flask once with water and refill the buret from a 250 c.c. Erlenmeyer flask containing the stock solution.
Measure 73.3 c.c. of the stock solution into the volumetric flask and dilute to 100 c.c. with distilled water.

Standards of Other Specific Gravities.—

Carry out the same procedure for preparation of the entire series down to 1.015, which covers the extreme ranges for blood and plasma. Use Table 16 to determine the amount of stock solution of gravity 1.100 to use.

TABLE 16.—CUBIC CENTIMETERS OF STOCK COPPER SULPHATE SOLUTION OF GRAVITY 1.100 TO BE DILUTED TO 100 C.C. TO PREPARE STANDARD GRAVITY SOLUTIONS*

GRAVITY	C.C.	GRAVITY	C.C.	GRAVITY	C.C.	GRAVITY	C.C.
1.075	74.30	1.060	59.00	1.044	43.00	1.029	28.06
1.074	73.30	1.059	58.00	1.043	42.00	1.028	27.08
1.073	72.20	1.058	57.00	1.042	41.00	1.027	26.10
1.072	71.20	1.057	56.00	1.041	40.00	1.026	25.12
1.071	70.20	1.056	55.00	1.040	39.00	1.025	24.14
1.070	69.10	1.055	54.00	1.039	38.00	1.024	23.15
1.069	68.10	1.054	53.00	1.038	37.00	1.023	22.17
1.068	67.00	1.053	52.00	1.037	36.00	1.022	21.19
1.067	66.00	1.052	51.00	1.036	35.00	1.021	20.20
1.066	65.00	1.051	50.00	1.035	34.00	1.020	19.21
1.065	64.00	1.050	49.00	1.034	33.00	1.019	18.22
1.064	63.00	1.049	48.00	1.033	32.00	1.018	17.23
1.063	62.00	1.048	47.00	1.032	31.00	1.017	16.24
1.062	61.00	1.046	45.00	1.031	30.00	1.016	15.25
1.061	60.00	1.045	44.00	1.030	29.04	1.015	14.26

*Plasma range—1.015 to 1.035.
Whole blood range—1.035 to 1.075.

Technic for Gravity Analysis of Blood and Plasma.—

Do not apply the tourniquet for more than one minute, when drawing whole blood. If desired, drops of whole blood may be delivered directly into the standards from the syringe and needle. Or finger blood may be used. Prepare the finger puncture, and take the blood up into a capillary tube. If the blood is transferred to a test tube containing anticoagulant, the cells and plasma must be thoroughly mixed immediately before a sample is drawn into a medicine dropper for the gravity test. For this purpose, (1) invert the tube ten times, or (2) raise and lower a glass rod with a mushroom end through the blood 10 times, just before the sample is drawn into the dropper.

Plasma gravity is best determined on plasma from blood which is treated with heparin, 0.2 mg. per c.c. of blood, as anticoagulant, since the heparin exerts no measurable effect on the gravity results. Or Heller and Paul's mixture of 3 parts of ammonium oxalate and 2 parts potassium oxalate may also be used for preparation of the blood. *Sodium citrate must not be used.*

Serum may be used in place of plasma. The gravity of serum indicates its protein content without the 0.2 or more gm. of fibrinogen per 100 c.c. removed by the clotting.

Deliver the drop of serum, plasma, or whole blood from a height of about 1 cm. above the solution from a medicine dropper, syringe needle, or capillary tube. When the drop is delivered, steady the dropper on the edge of the bottle.

The delivered drop breaks through the surface film of the solution and then penetrates 2 to 3 cm. below the surface; within five seconds the momentum of the fall is lost and the drop then either begins to rise, or becomes stationary, or continues to fall. The gravity of the drop relative to the solution does not change appreciably until the drop has been immersed in the solution for another 10 to 15 seconds, and there is ample time to note its behavior during this interval. *If the drop is lighter than the test solution, it will rise, perhaps only*

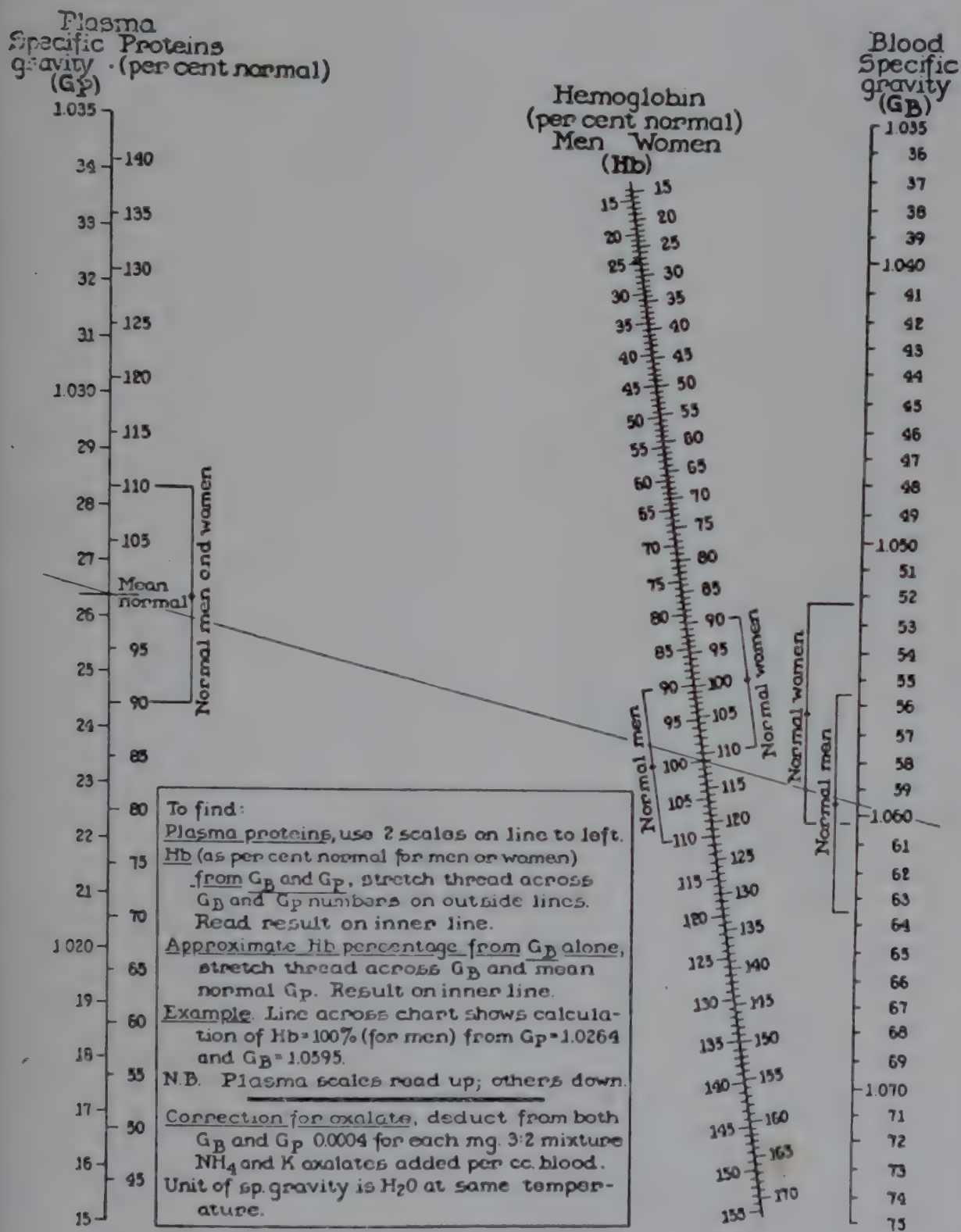


Fig. 109.—Line chart for calculating percentages of normal plasma proteins and hemoglobin from gravities of plasma and blood. (Courtesy U. S. Navy Research Unit, Hospital of the Rockefeller Institute for Medical Research, New York City, by permission of the Surgeon General, U. S. Navy.) (From Simmons and Gentzkow: Laboratory Methods of the United States Army, ed. 5, Philadelphia, 1944, Lea & Febiger. Reprinted by permission.)

a few millimeters, and may begin to sink immediately afterward. *If the drop is of the same gravity as the standard test solution, it will become stationary for this interval and then fall. If the drop is heavier, it will continue to fall during this interval. It is to be emphasized that the behavior during the ten seconds after the drop has lost momentum of its fall into the solution indicates whether the drop is lighter or heavier than the test solution—if it rises at all during this period, it is lighter than the standard.*

For the test, add a drop of the blood, serum, or plasma to various standards and select as the reading the standard which is of the same specific gravity as the blood, that is, where the drop neither rises nor falls but remains stationary.

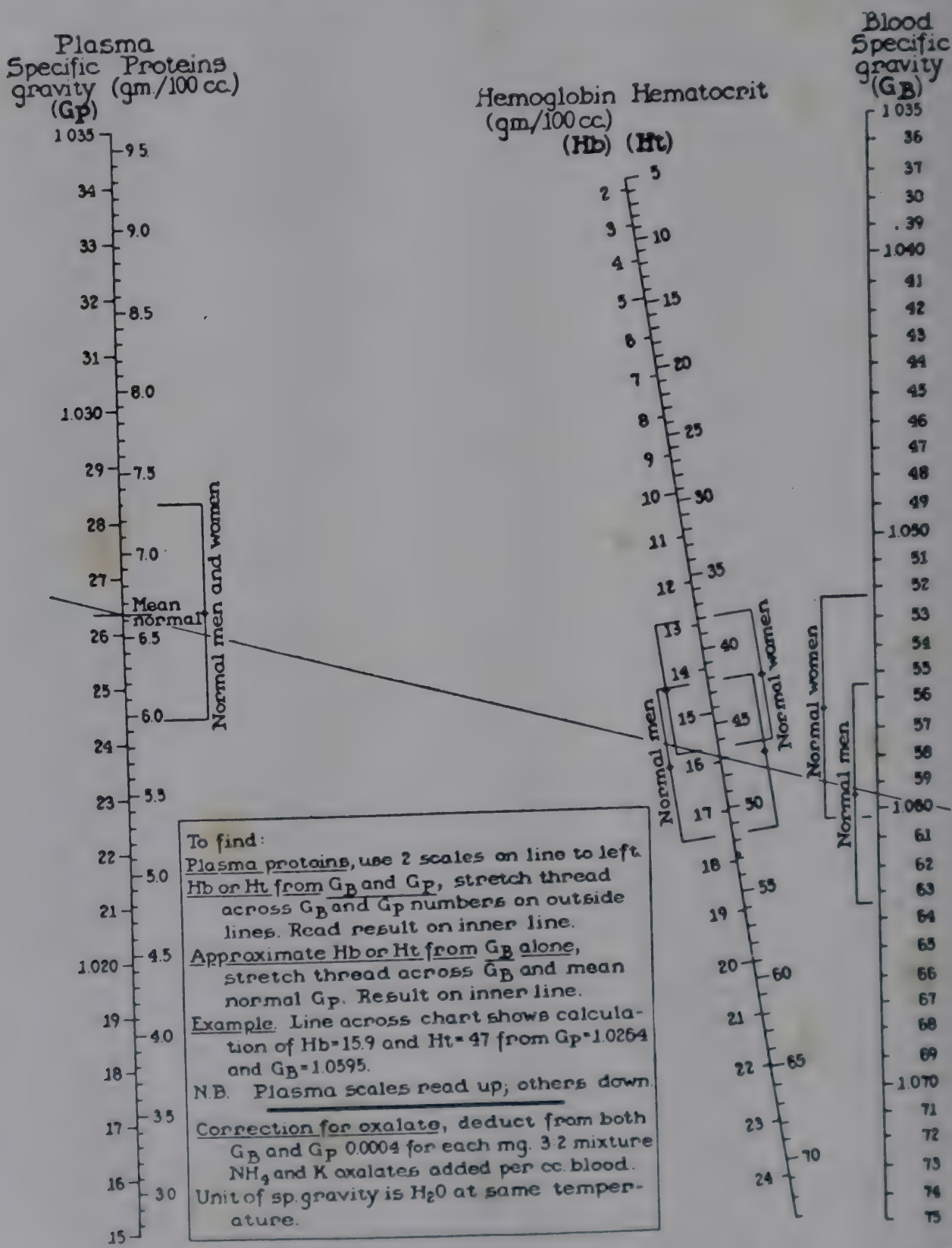


Fig. 110.—Line chart for calculating plasma proteins, hemoglobin, and hematocrit from gravities of plasma and blood. (Courtesy U. S. Navy Research Unit, Hospital of the Rockefeller Institute for Medical Research, New York City, by permission of the Surgeon General, U. S. Navy.) (From Simmons and Gentzkow: Laboratory Methods of the United States Army, ed. 5, Philadelphia, 1944, Lea & Febiger. Reprinted by permission.)

Calculation.—

Calculations are made with lined charts which take into account plasma protein concentration, hemoglobin concentration in blood, and hematocrit percentages. Hemoglobin and hematocrit values are estimated by laying a straightedge across the charts. See Figs. 109 and 110.

Accuracy of the Method.—

The hemoglobin content by the gravity method has been found regularly within 2 per cent of the correct value measured by oxygen capacity. Usually the accuracy is within ± 0.2 gm. In pathologic blood conditions, such as pernicious anemia, the error can be larger.

This method has been used widely in official circles and has been definitely accepted.

The Falling Drop Method for Specific Gravity of Protein*

This is a method for determination of specific gravity of blood and plasma and from this a determination of serum protein and plasma protein can be made. The apparatus recommended are the Kagan proteinometer, manufactured by E. H. Sargent Co., Chicago, and that manufactured by Eimer and Amend (Fig. 111).

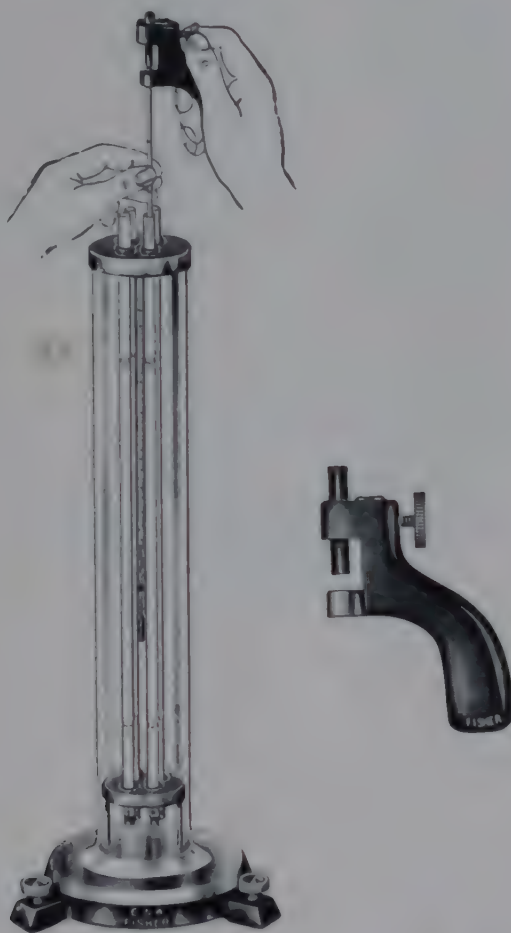


Fig. 111.

Fig. 112.

Fig. 111.—The Eimer and Amend falling drop apparatus. (Courtesy Fisher Scientific Co., Pittsburgh.)

Fig. 112.—Guthrie pipette controller. (Courtesy Fisher Scientific Co., Pittsburgh.)

The blood of a normal male has a specific gravity of 1.0566. When the heavy blood cells are separated from the plasma by centrifuging, the latter is found to have a specific gravity of 1.0270. These weights, together with the normal red blood cell count of 5,600,000 per cubic millimeter and the normal cell volume of 46 per cent as determined by hematocrit, are characteristic of normal bodily functions.

*Barbour and Hamilton: J. Biol. Chem. 69: 625-640, 1926.

This method enables one to determine the specific gravity of a single drop of blood by comparing its time of fall through a liquid with the time of fall required by a liquid of known specific gravity and referring this information to a simplified alignment chart supplied with the apparatus. If the percentage of plasma protein in blood plasma is desired, the specific gravity of the plasma is observed at 20° C., and this figure is readily converted to a percentage by referring to a printed table also supplied with the apparatus.

The apparatus consists of a tall glass cylindrical water bath which is permanently mounted upon a base equipped with leveling screws (Fig. 111).

There are four falling drop tubes suspended from the tight-fitting cover. These tubes are open at both ends and fitted with cork stoppers in order to facilitate cleaning. The tubes are labeled B₁, B₂, P₁, and P₂. From a hook in the center of the cover hangs an accurately graduated thermometer, which serves as a plumb bob as well as a means of determining the temperature of the water bath. The water bath is used to insure uniform temperature of the standard solutions and of the unknown being tested.

The principle of this determination is based upon laboratory data showing that within instrumental limits of error, the protein content of either is directly proportional to the specific gravity of the serum or plasma.

Both plasma protein and serum protein may be figured from the specific gravities.

Plasma protein is determined by using the formula

$$(\text{plasma sp. gr.} - 1.0069) \times 340.1.$$

The serum protein is determined by using the formula

$$(\text{serum sp. gr.} - 1.0073) \times 347.9.$$

Fill the falling drop tubes, properly labeled, with appropriate liquids which are immiscible with the body fluids. Fill tube B₁ with a meta-xylene 70.5% and bromobenzene 29.5% mixture, and use for normal and anhydremic blood. This solution has a specific gravity of 1.053. Use B₂ also for normal and anhydremic blood; it has a specific gravity of 1.043, and is a mixture of meta-xylene 72.1%, bromobenzene 27.9%. P₁ is a mixture of meta-xylene 75.3% and bromobenzene 24.7% with a specific gravity of 1.023; use it for anemic blood and heavy secretions. P₂ is meta-xylene 76.9% and bromobenzene 23.1% with a specific gravity of 1.013; use it for plasma, serum, transudates, heavy urine, and extremely anemic blood.

General Procedure.*—

By means of the Guthrie pipette controller (Fig. 112), designed by C. C. Guthrie, School of Medicine, University of Pittsburgh, release a drop containing 0.01 c.c. of body fluid into the falling drop tube filled with a liquid immiscible with the body fluid. Time the drop as it passes between the two marks, 30 cm. apart, engraved upon the tube. Use a stop watch calibrated in $\frac{1}{10}$ seconds and note the time. Record the temperature of the water bath.

Find the apparent density difference of the body fluid by means of the alignment chart furnished with each set. Determine the apparent density difference of the standard solution of known specific gravity by going through similar operations as already described.

With the above data and by applying simple arithmetic, find the specific gravity of the body fluid.

*The author is indebted to Fisher Scientific Company, Pittsburgh, Pa., for permission to reproduce cuts and information published by Eimer and Amend, 205-223 Third Ave., New York City.

Determination of the Specific Gravity of Whole Blood

Mount falling drop pipette in pipette holder as shown in Fig. 113.

Turn thumbscrew of the pipette holder all the way in.

Puncture finger; wipe away first drop; draw up blood slightly above second mark of pipette by turning thumbscrew.

The whole blood is used just as it issues from the patient without addition of any anticoagulant or reagent.

Wipe tip of pipette dry and bring the top of the column of blood even with the middle mark.

Insert tip of pipette in a vertical position not more than $\frac{1}{4}$ inch below surface of mixture in falling drop tube.

Use Tube B₁ (sp. gr. 1.0530) for normal and concentrated bloods.

Use Tube B₂ (sp. gr. 1.0430) for anemic bloods.



Fig. 113.—Delivering the drop. One hand holds the pipette; the other releases the sample. (Courtesy Fisher Scientific Co., Pittsburgh.)

Form the drop by turning the thumbscrew until the top of the blood column falls to the lower mark.

Release the drop by gently removing the tip of the pipette from the tube.

Start the stop watch as the center of the drop passes the upper mark.

Stop the stop watch as the center of the drop passes the lower mark.

Record the falling times.

Repeat the procedure using another finger if the blood is not flowing freely from the first puncture; use a clean pipette. Two drops of whole blood should not be dropped from the same pipette, for the second drop is always heavier as the result of sedimentation of the cells in the interval during which the first drop is falling.¹ Stop watch must register $\frac{1}{10}$ second. Do not attempt timing with a $\frac{1}{5}$ second stop watch.

Average the observed falling times.

Note: If the blood falls slower than 100 seconds in Tube B₁, repeat, using falling drop Tube B₂. If the blood falls faster than 20 seconds, it should be repeated in a falling drop tube containing a denser solution.

¹Drew, Scudder, and Rapps: Surg., Gynec. & Obst. 70: 859, 1940.

Determination of the Specific Gravity of Plasma or Serum

Preparation of the Plasma.—

Draw blood from vein with a sterile dry syringe without the use of a tourniquet if possible; or, if a tourniquet is used, release it and wait a minute for blood to recirculate before drawing the sample.

Gently introduce from 5 to 6 c.c. into a Sanford-Magath hematocrit tube containing the proper amount of heparin (about 1 mg.). Avoid air bubbles.

Gently invert the tube two or three times to mix the blood with the anticoagulant.

Cork with rubber stopper (no-air No. 5), counterbalance, and spin in centrifuge for 1 hour at 2,500 revolutions a minute (in emergency cases 15 minutes is sufficient).¹

Preparation of the Serum.—

Follow usual procedure for obtaining serum.

Specific Gravity of Plasma or Serum.—

The steps for determining specific gravity of plasma or serum are exactly the same as those described for whole blood, except that 2 drops are used from each pipette and No. III standard (specific gravity 1.0268). The determination is easier because there is no tendency toward coagulation.

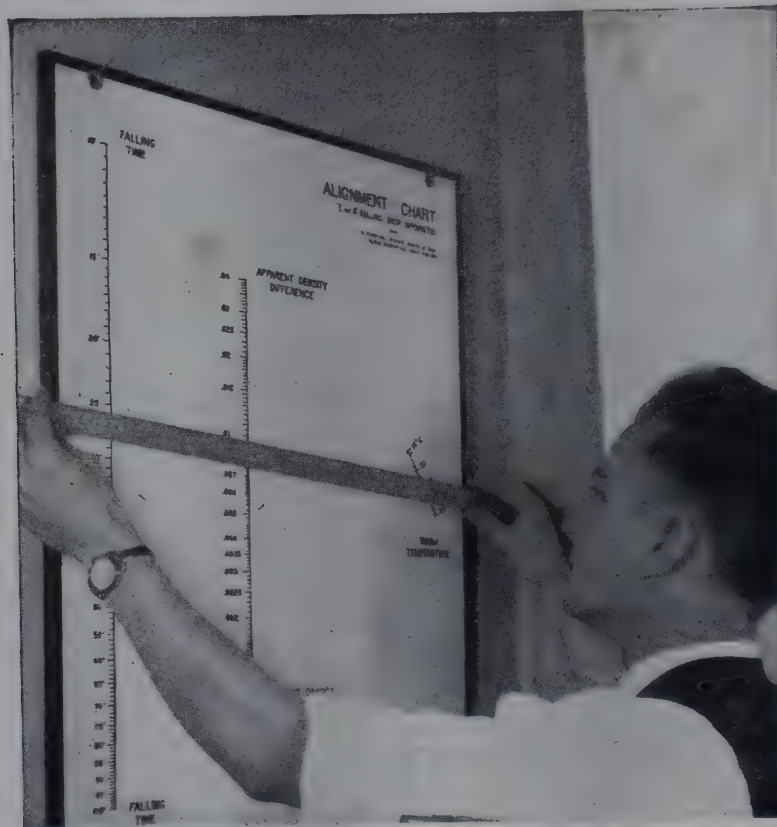


Fig. 114.—Large alignment chart for accurate readings corrected to the temperature in the water jacket. (Courtesy Fisher Scientific Co., Pittsburgh.)

For heavy plasma or serum, Tube P₁ is used (specific gravity 1.0230); for lighter plasma or serum, tube P₂ (specific gravity 1.0130). Again the falling time of the plasma or serum is checked against the falling time of the standard and the true density difference is added or subtracted to the corrected specific gravity of the standard.¹

Calculation of Specific Gravity of Plasma or Serum or Whole Blood.

1. Place a ruler between the falling time of the standard drop and the temperature of the bath, and record the apparent density difference where the ruler intersects the chart. (Use the chart supplied with the apparatus, Fig. 114.)
2. Follow the same procedure for the drop of plasma, serum, or whole blood.
3. Subtract the smaller apparent density difference from the larger, which gives the difference in specific gravity between the unknown and the standard.

¹Drew, Scudder, and Rapps: Surg., Gynec. & Obst. 70: 859, 1940.

4. Correct the specific gravity of the standard to the temperature of the bath by *subtracting 0.0001 for every 2 degrees rise* in temperature above 20° C., or by *adding 0.0001 for every 2 degrees below 20° C.*

5. If the falling time of the blood is greater than that of the standard, the drop is lighter, and the difference found in the third step is *subtracted* from the corrected standard specific gravity.

6. If the falling time of the blood is less than that of the standard, the blood is heavier, and the difference is *added* to the corrected specific gravity of the standard.

To Convert Specific Gravity to Plasma Protein.—

Determine the plasma protein content from the specific gravity by reference to Table 17, or use the following formula:

$$\text{Plasma Protein} = (\text{Plasma sp. gr.} - 1.0069) \times 340.1.$$

To Convert Specific Gravity to Serum Protein.—

Determine the serum protein content from the specific gravity by reference to Table 18, or use the following formula:

$$\text{Serum Protein} = (\text{Serum sp. gr.} - 1.0073) \times 347.9.$$

Criteria for Checks on Falling Time.—

When a drop of blood, plasma, serum, or standard potassium sulphate solutions falls in the bromobenzene mixture, successive drops from the same or different pipettes should give apparent density differences of not greater than 1×10^{-4} . It will be observed that 0.1 of a second in the rapid range of falling time constitutes a larger range in the apparent density differences than 0.5 of a second difference in the falling time of 2 successive drops at the other end of the scale. When accurate checks cannot be made, a sufficient number of drops should be done to establish a good average falling time.¹

Reasons for Inability to Get Checks on Drops.—

1. Dirty pipettes cause drops of varying sizes to be delivered.
2. Slipping drop due to rubber tubing in pipette holder being too large causing incomplete suction.
3. Too much tension on thumbscrew at moment of release causes rebound of fluid in pipette when drop is released. Thumbscrew should compress rubber tubing in holder uniformly and thumb should be removed from the screw before pipette is lifted from tube so that level of fluid in pipette remains at the mark.
4. Torsion of hand steadying tip of pipette increases pressure in rubber tube and, therefore, a rebound suction action occurs at release. Relieve all tension before drop is released.
5. Currents in the dropping tube due to rapidly changing temperature on one side of the water jacket; e.g., that caused by opening a window or lighting a burner near the apparatus.
6. Air bubble or particle of solid material.
7. Poor reaction time in using stop watch. A stop watch stand will aid in accurate timing.
8. Incomplete mixture of the bromobenzene xylene solution causing varying rates of speed in the falling drop.
9. Gross differences in temperature between bromobenzene mixture and plasma. These are minor technical details which are quickly mastered but which in the beginning give trouble.¹

Details of Technic—Precautions

Adjustment of Falling Drop Tubes.—

See that rubber collar at top of the falling drop tube is square across.

The falling drop tubes should be kept scrupulously clean. After repeated use, should the mixture in the tube become opalescent, the tube should be cleaned and new mixture added.

Make certain the falling drop tubes are hanging plumb (thermometer serves as a plumb bob). Adjustment is made with leveling screws in base.

¹Drew, Scudder, and Rapps: Surg., Gynec. & Obst. 70: 859, 1940.

If drops of standard, plasma, or blood adhere to sides, tube should be cleaned with the cleaning rod.

Manipulation of Falling Drop Pipette.—

Guide pipette by holding lower part with fingers of left hand (see Fig. 113), resting left hand, for steadying purposes, on top of cylindrical vessel. Manipulate the thumb-screw with the right hand.

The drop, when released, must travel down the center of the falling drop tube.

Note: If drop is released too far off center it will be drawn to the side, probably smearing it, and necessitating the cleaning of the falling drop tube.

The pipette must be in a vertical position, approximately plumb, to insure that uniform drops are released.

Pipettes should be cleaned immediately after use by flushing with ammonia water, water, alcohol, ether, and air.

Preservation of Standards.—

These should be kept under mineral oil. Small portions may be removed during the day and kept in glass stoppered bottles for routine use.

Evaporation is to be guarded against at all cost. The appearance of a mold in the standard necessitates its rejection.

Uses of the Standard Potassium Sulphate Solutions

The following table is suggestive only. The standard solution need not be exactly of the specific gravity indicated. See "Specific Gravity of Potassium Sulphate Solutions." Other standards can, of course, be made up to meet the users' requirements.

Potassium Sulphate Solutions

USE	SPECIFIC GRAVITY AT 20° C.	GRAMS K ₂ SO ₄ PER
		1000 C.C. H ₂ O AT 20° C.
A. Cerebrospinal fluid, dilute urine, secretions and exudates	1.0000	00.00
B. Dilute plasma, serum, transudates, heavy urine, extremely anemic blood, etc.	1.0150	18.84
C. Plasma and serum	1.0269	33.91
D. Anemic blood	1.0350	44.56
E. Normal and anhydremic blood	1.0550	70.81

Note: The specific gravities are based on water at the same temperature as the solution.

How to Mix the Meta-Xylene-Bromobenzene Solutions*

(The percentages are by volume)

- No. 1. m-xylene 80.0%, bromobenzene 20.0% = 0.993 sp. gr.
- No. 2. m-xylene 78.5%, bromobenzene 21.5% = 1.003 sp. gr.
- No. 3. m-xylene 76.9%, bromobenzene 23.1% = 1.013 sp. gr.
- No. 4. m-xylene 75.3%, bromobenzene 24.7% = 1.023 sp. gr.
- No. 5. m-xylene 73.7%, bromobenzene 26.3% = 1.033 sp. gr.
- No. 6. m-xylene 72.1%, bromobenzene 27.9% = 1.043 sp. gr.
- No. 7. m-xylene 70.5%, bromobenzene 29.5% = 1.053 sp. gr.

Uses of Above Meta-Xylene-Bromobenzene Solutions

- No. 1. Cerebrospinal fluid, dilute urine, secretions, and exudates.
- Nos. 2 and 3. Plasma, serum, transudates, heavy urine, extremely anemic blood, etc.
- Nos. 4 and 5. Anemic blood and heavy secretions.
- Nos. 6 and 7. Normal and anhydremic blood.

*Only Eastman Kodak No. 43 bromobenzene and T-275 m-xylene should be employed in making these mixtures.

TABLE 17.—TRANSLATION OF PLASMA SPECIFIC GRAVITY INTO PLASMA PROTEIN
ACCORDING TO WEECH'S FORMULA*

$$\text{Plasma Protein} = (\text{Plasma sp. gr.} - 1.0069) \times 340.1$$

$$\text{Serum Proteins} = (\text{Serum sp. gr.} - 1.0073) \times 347.9$$

These values were calculated at 20° C.

1.0187	4.00%	1.0255	6.32%	1.0323	8.65%
1.0188	4.04	1.0256	6.36	1.0324	8.68
1.0189	4.08	1.0257	6.40	1.0325	8.71
1.0190	4.12	1.0258	6.43	1.0326	8.75
1.0191	4.15	1.0259	6.46	1.0327	8.78
1.0192	4.18	1.0260	6.50	1.0328	8.81
1.0193	4.22	1.0261	6.53	1.0329	8.85
1.0194	4.25	1.0262	6.56	1.0330	8.89
1.0195	4.28	1.0263	6.60	1.0331	8.92
1.0196	4.32	1.0264	6.63	1.0332	8.95
1.0197	4.35	1.0265	6.66	1.0333	8.99
1.0198	4.39	1.0266	6.70	1.0334	9.02
1.0199	4.42	1.0267	6.73	1.0335	9.05
1.0200	4.45	1.0268	6.76	1.0336	9.09
1.0201	4.48	1.0269	6.80	1.0337	9.12
1.0202	4.52	1.0270	6.83	1.0338	9.15
1.0203	4.56	1.0271	6.86	1.0339	9.19
1.0204	4.60	1.0272	6.90	1.0340	9.23
1.0205	4.63	1.0273	6.94	1.0341	9.26
1.0206	4.66	1.0274	6.97	1.0342	9.30
1.0207	4.69	1.0275	7.01	1.0343	9.33
1.0208	4.72	1.0276	7.04	1.0344	9.36
1.0209	4.76	1.0277	7.07	1.0345	9.40
1.0210	4.79	1.0278	7.11	1.0346	9.43
1.0211	4.83	1.0279	7.14	1.0347	9.46
1.0212	4.86	1.0280	7.17	1.0348	9.50
1.0213	4.90	1.0281	7.21	1.0349	9.53
1.0214	4.93	1.0282	7.24	1.0350	9.56
1.0215	4.96	1.0283	7.28	1.0351	9.60
1.0216	4.99	1.0284	7.32	1.0352	9.64
1.0217	5.03	1.0285	7.35	1.0353	9.67
1.0218	5.07	1.0286	7.38	1.0354	9.70
1.0219	5.11	1.0287	7.42	1.0355	9.74
1.0220	5.14	1.0288	7.45	1.0356	9.77
1.0221	5.17	1.0289	7.48	1.0357	9.80
1.0222	5.21	1.0290	7.52	1.0358	9.84
1.0223	5.24	1.0291	7.55	1.0359	9.87
1.0224	5.27	1.0292	7.58	1.0360	9.90
1.0225	5.31	1.0293	7.62	1.0361	9.94
1.0226	5.34	1.0294	7.65	1.0362	9.97
1.0227	5.37	1.0295	7.68	1.0363	10.00
1.0228	5.41	1.0296	7.72	1.0364	10.04
1.0229	5.44	1.0297	7.75	1.0365	10.07
1.0230	5.48	1.0298	7.78	1.0366	10.10
1.0231	5.52	1.0299	7.83	1.0367	10.13
1.0232	5.54	1.0300	7.86	1.0368	10.17
1.0233	5.58	1.0301	7.89	1.0369	10.21
1.0234	5.62	1.0302	7.93	1.0370	10.23
1.0235	5.66	1.0303	7.96	1.0371	10.27
1.0236	5.69	1.0304	8.00	1.0372	10.31
1.0237	5.72	1.0305	8.03	1.0373	10.34
1.0238	5.75	1.0306	8.06	1.0374	10.37
1.0239	5.78	1.0307	8.10	1.0375	10.41
1.0240	5.81	1.0308	8.13	1.0376	10.44
1.0241	5.85	1.0309	8.16	1.0377	10.47
1.0242	5.89	1.0310	8.20	1.0378	10.51
1.0243	5.92	1.0311	8.24	1.0379	10.55
1.0244	5.95	1.0312	8.27	1.0380	10.58
1.0245	5.99	1.0313	8.30	1.0381	10.61
1.0246	6.02	1.0314	8.34	1.0382	10.65
1.0247	6.05	1.0315	8.37	1.0383	10.68
1.0248	6.09	1.0316	8.40	1.0384	10.71
1.0249	6.12	1.0317	8.44	1.0385	10.75
1.0250	6.15	1.0318	8.47	1.0386	10.79
1.0251	6.19	1.0319	8.50	1.0387	10.82
1.0252	6.22	1.0320	8.54	1.0388	10.86
1.0253	6.25	1.0321	8.57	1.0389	10.89
1.0254	6.29	1.0322	8.61	1.0390	10.92

*J. Biol. Chem. 113: 167, 1936.

The Use of Heparin

The effect upon the specific gravity and other physical properties of the blood when heparin is employed is practically negligible, whereas such reagents as sodium oxalate are likely to change the specific gravity, and also influence other physical properties to an extent that might prevent the use of the same blood sample for other tests.

For a 6 c.c. sample of blood, a small amount, such as can be picked up on the tip of a penknife blade, suffices (about 1 mg.).

TABLE 18.—APPROXIMATE BLOOD VALUE

SPECIFIC GRAVITY	HEMOGLOBIN	HEMATOCRIT PER CENT CELLS	RED BLOOD COUNT
1.030	0	0	0
1.035	20	10	1,000,000
1.040	40	20	2,000,000
1.045	60	30	3,000,000
1.050	80	40	4,000,000
1.055	100	50	5,000,000
1.060	120	60	6,000,000

From Guthrie, C. C.: J. Lab. & Clin. Med. 17: 1158, 1932.

Specific Gravity of Potassium Sulphate Solutions

It is extremely important that the *correct* specific gravity of each standard potassium sulphate solution be known. It is *not* important that the specific gravity of each solution agree in the fourth decimal place with the figures as given by the authors, because the falling drop method resorts to a comparative test in which the falling time of a solution of known specific gravity is compared with that of a solution of unknown specific gravity.

Eimer and Amend standard potassium sulphate solutions are made and then standardized in their laboratories in the most painstaking manner, and the correct specific gravity of each solution is noted upon the label of the bottle.

The specific gravity of one lot of standard potassium sulphate solution is likely to vary in the fourth decimal place from the specific gravity of another lot.

X-B Solutions as Used at Columbia

At the College of Physicians and Surgeons the solutions used in the falling drop tubes which are engraved B₁, B₂, P₁, and P₂, respectively, are as follows:

- B₁, solution No. 7, sp. gr. 1.053 Normal and anhydremic blood.
- B₂, solution No. 6, sp. gr. 1.043 Normal and anhydremic blood.
- P₁, solution No. 4, sp. gr. 1.023 Anemic blood and heavy secretions.
- P₂, solution No. 3, sp. gr. 1.013 Plasma, serum, transudates, heavy urine, extremely anemic blood, etc.

Note: Solutions Nos. 3, 4, 6, and 7 need only be approximately of the above-mentioned specific gravities.

Determinations of Proteins in Small Amounts of Blood Serum and Plasma,
Weichselbaum Method¹

A. Total Protein Determination by Visual Colorimetry.—

Reagents.—

30% Urea Thymol Solution.—

- Dissolve 300 gm. of c.p. or U.S.P. urea in approximately 700 c.c. of distilled water.
- Add a crystal of thymol about the size of a green pea.
- Heat over a free flame.
- Add 3 gm. of norite and boil for a few minutes.
- Filter.
- Dilute to 1,000 c.c. with distilled water.
- The use of the charcoal is to prevent cloudiness on standing.

¹Am. J. Clin. Path.: 7: 140, 1946.

Protein Standards.—

(A) Use clear pooled human or animal blood serum.

Determine the total protein content by a macro-Kjeldahl method.

Measure into a 250 c.c. volumetric flask a quantity of the pooled serum containing 0.9 gm. of protein.

Dilute to 250 c.c. with the urea-thymol solution.

This standard is stable at room temperature for at least 12 months.

100 c.c. contain 360 mg. of protein (9 gm. per 100 c.c. protein based on the use of 0.2 c.c. sample of serum and a volume of 10 c.c.).

(B) Proceed as for standard A except measure a volume of serum containing 0.7 gm. protein into the 250 c.c. volumetric flask.

100 c.c. contain 280 mg. protein (7 gm. protein per 100 c.c. based on the use of a 0.2 c.c. sample of serum and a total volume of 10 c.c.).

(C) Proceed as for standard A except to measure a volume of serum containing 0.4 gm. protein into the 250 c.c. volumetric flask.

100 c.c. contain 160 mg. protein (4 gm. protein per 100 c.c.).

Biuret Reagent.—

Sodium potassium tartrate ----- 45 gm.

Copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) ----- 15 gm.

Potassium iodide (KI) ----- 5 gm.

0.2 N Sodium hydroxide (carbonate free).

Dissolve the Rochelle salt in approximately 400 c.c. of 0.2 N sodium hydroxide.

Add the copper sulphate and dissolve *completely* with stirring.

Add the potassium iodide.

Dilute to 1,000 c.c. in a volumetric flask, using 0.2 N sodium hydroxide as the diluent.

Technic.—

With a Folin-Ostwald pipette (to contain), measure 0.2 c.c. of blood serum into a test tube containing 4.8 c.c. of urea thymol solution.

With a 5 c.c. volumetric pipette, measure 5 c.c. portions of the working standards A, B, C into similar tubes.

Into each tube, measure 5 c.c. of biuret reagent.

Mix well by whirling.

Place in a 40° to 50° C. water bath for 5 to 10 minutes.

Remove, and allow to cool to room temperature.

If there is no particular hurry, the tubes may be allowed to stand at room temperature for 30 minutes for full development of color. There is no fading or clouding of the colors on standing for as long as 5 days.

Compare in a Duboseq type colorimeter with the standard the color of which is closest to the unknown, or, for more accurate results, if the unknown varies more than 15% from the standard used, compare also with the next closest standard.

Calculations.—

(1) Unknown read against a single standard:

$$\frac{R_s}{R_x} \times \text{gm. of protein in 5 c.c. standard} \times \frac{100}{0.2} = \text{gm. of protein per 100 c.c.}$$

R_s , reading of standard; R_x , reading of unknown.

The gm. of protein in 5 c.c. of standard is:

$$A = 0.018, B = 0.014, C = 0.008.$$

Simplified to

$$\frac{R_s}{R_x} \times \begin{matrix} 9 \text{ (standard A)} \\ 7 \text{ (standard B)} \\ 4 \text{ (standard C)} \end{matrix} = \text{gm. protein per 100 c.c.}$$

(2) Unknown read against nearest two standards:

$$\frac{\frac{A}{B} \frac{R_s}{R_x} + \frac{A}{C} \frac{R_s}{R_x}}{2}$$

Example: Standards B and C are the closest two samples.

$$\frac{7^B(R_s/R_x) + 4^C(R_s/R_x)}{2}$$

B. Total Protein Determination by Spectrophotometry.—

Reagents.—

0.85% Sodium Chloride.—

Dissolve 8.5 gm. sodium chloride, c.p., in distilled water, and dilute to 1,000 c.c. with distilled water. Filter.

Biuret Reagent.—See above.

Technic.—

Place 4.9 c.c. of 0.85% sodium chloride in a cuvette.

Add 0.10 c.c. of blood serum from a Folin-Ostwald pipette (to contain).

Measure 5 c.c. of the biuret reagent into the cuvette.

Mix well by whirling.

Allow to stand in a water bath at 30° to 32° C. for approximately 30 minutes for full color development.

Prepare a reference "blank" solution at the same time:

Add 5 c.c. of the biuret solution to 5 c.c. of 0.85% sodium chloride in a similar cuvette and incubate as above.

Read the solutions in a spectrophotometer as soon as possible after removal from the water bath, at 555 mμ.

Temperature has a slight effect on the intensity of the color of the biuret complex, but variations of temperature from 28° to 34° C. are not sufficient to cause appreciable errors.

The gram percentage of total serum protein is read directly from an extinction (optical density)-concentration curve, which may be constructed by the use of the pooled human or animal serum (see protein standards, reagents).

Dilute the Kjeldahl-determined serum with 0.85% sodium chloride for the construction of this curve instead of using urea thymol.

C. Serum Albumin Determination by Visual Colorimetry.—

Reagents.—

23% Sodium Sulphate.—

Dissolve 230 gm. of anhydrous sodium sulphate in distilled water in a 1,000 c.c. volumetric flask and dilute to 1,000 c.c. with distilled water. Filter. Keep in a warm place in the laboratory, or preferably in an incubator to prevent crystallization.

Ether, Ethyl, C.P. or U.S.P.—

Methyl Alcohol Reagent.—

Add 607 c.c. of c.p. methyl alcohol
to 393 c.c. of distilled water.

Cool to 0° C.

Dilute to 1 liter with cold methyl alcohol.

Acetate Buffer.—

Mix 72 c.c. of 1 M acetic acid
and 12 c.c. of 1 M sodium hydroxide
and dilute to 1 liter with distilled water.

Technic.—**(1) Precipitation of Globulin Fraction by Sodium Sulphate:**

Using a Folin-Ostwald pipette (to deliver), measure 0.5 c.c. of serum into a 15 c.c. centrifuge tube.

Add 7.5 c.c. of 23% sodium sulphate
and 3 c.c. of ether.

Stopper with a tightly fitting rubber stopper and shake vigorously for approximately 30 seconds.

Centrifuge at approximately 2,000 r.p.m. for 5 minutes.

Draw off the albumin layer as follows: Carefully tip the centrifuge tube until the globulin layer breaks away from the tube wall.

Introduce a 5 c.c. volumetric pipette, holding the index finger over the top to prevent the entrance of the ether layer, and being careful not to break up the globulin layer.

Insert the pipette to the bottom of the tube and draw off 5 c.c. of clear albumin solution, being careful to wipe the outside of the pipette free from any solid particles of protein with filter paper before adjusting exactly to the mark.

Proceed exactly as for the determination of total protein.

(2) Precipitation of Globulin Fraction by Buffered Methyl Alcohol:

Maintain the serum and all the reagents at all times between 0° and 1° C. by using a small ice bath.

Measure 2 c.c. of fresh serum into a 15 c.c. centrifuge tube.

Add 1 c.c. of acetate buffer, with stirring, maintaining the temperature at 0° to 1° C.

Add 7 c.c. of cold methyl alcohol reagent, with stirring.

Mix thoroughly.

Allow the tube to stand in a water bath at 0° C. for 30 minutes.

The globulin will have precipitated, while the albumin remains in solution.

Chill a small filter funnel fitted with a fluted No. 42 Whatman filter paper by placing in an icebox.

Filter the cold methyl alcohol protein suspension at room temperature, provided that the 2 c.c. aliquot of filtrate needed for the analysis can be obtained within 5 to 7 minutes after removal of the material from the cold.

Measure a 2 c.c. aliquot of the filtrate into a tube containing 3 c.c. of urea thymol.

Proceed exactly as for the determination of total protein.

Calculations.—

(1) Unknown from the sodium sulphate globulin precipitation read against a single standard:

$$\frac{R_s}{R_x} \times \text{gm. amount of protein in 5 c.c. standard} \times \frac{100}{0.3125}$$

Simplified to:

$$\frac{R_s}{R_x} \times \frac{5.76 \text{ (A)}}{4.48 \text{ (B)}} = \text{gm. albumin per 100 c.c.}$$

$$\frac{R_s}{R_x} \times \frac{2.56 \text{ (C)}}{4.48 \text{ (B)}}$$

(2) Unknown from methyl alcohol globulin precipitation read against a single standard:

$$\frac{R_s}{R_x} \times \text{gm. of protein in 5 c.c. standard} \times \frac{100}{0.4}$$

Simplified to:

$$\frac{R_s}{R_x} \times \frac{4.5 \text{ (A)}}{3.5 \text{ (B)}}$$

$$\frac{R_s}{R_x} \times \frac{2.0 \text{ (C)}}{3.5 \text{ (B)}}$$

For comparisons of the unknown albumin with the nearest two standards, the formula given under total proteins is applied.

Total Globulin.—

Subtract the results of the serum albumin determination from the results of the total protein to obtain the total globulin.

D. Serum Albumin Determination by Spectrophotometry.—

(1) This procedure is identical with the visual colorimetric description through the obtaining of the 5 c.c. aliquot of the clear albumin solution.

To this 5 c.c. aliquot

add 5 c.c. of 0.85% sodium chloride.

Mix well.

Add 10 c.c. of biuret reagent.

Mix well.

Allow to stand 30 minutes in a water bath at 30° to 32° C.

Prepare a reference "blank" solution by adding 5 c.c. of 0.85% sodium chloride solution to 5 c.c. of 23% sodium sulphate.

Add 10 c.c. of biuret reagent, and incubate as above.

Read the serum albumin solution against the reference "blank" in a spectrophotometer at 555 m μ .

The optical density value is located on the total protein optical density-concentration curve and the value obtained is divided by 1.562 to give gm. % serum albumin.

(2) This procedure is identical with serum albumin determination by visual colorimetry except that a 1 c.c. aliquot of methyl alcohol filtrate is added to 4 c.c. of saline and a reference "blank" containing 1 c.c. of methyl alcohol reagent and 4 c.c. of saline and 5 c.c. of biuret reagent is employed.

The optical density reading is located on the total protein optical density-concentration curve and the value obtained is divided by 2 to give gm. albumin per 100 c.c.

Normal Values.—

Total Protein: 6.5 to 7.7; average, 7.14 gm. per 100 c.c.

Albumin: 3.9 to 4.6; average, 4.19 gm. per 100 c.c.

Globulin: 2.3 to 3.5; average, 2.9 gm. per 100 c.c.

Globulin and Albumin in Blood Serum, Photoelectric Method of Looney and Walsh¹

The method of Looney and Walsh for the determination of the albumin and globulin content of blood serum with the photoelectric colorimeter is a very simple and useful procedure. It embodies the precipitation of a 1 per cent dilution of serum with sulphosalicylic acid in the presence of gum ghatti. The globulin is determined directly in a 10 per cent dilution by precipitation with one-half saturation of ammonium sulphate in the presence of the same protective colloid. The method is applicable to 0.2 c.c. of serum and can be carried out in 5 to 10 minutes.

Reagents—

1% Salt Solution.—

Dissolve 1 gm. sodium chloride in 100 c.c. distilled water.

2% Gum Ghatti.—

See page 245.

Saturated Solution of Ammonium Sulphate.—

See page 307. Reaction should be pH 6.5.

5% Sulphosalicylic Acid.—

Dissolve 5 gm. sulphosalicylic acid in enough distilled water to make 100 c.c. solution.

Serum Globulin Solutions.—

To make the standard curve, prepare different dilutions of serum globulin of known concentrations.

¹Looney, J. M., and Walsh, A. E.: J. Biol. Chem. 130: 635-639, 1939.

Treat serum² with an equal volume of saturated ammonium sulphate of pH 5.6. Dialyze this material in a collodion or cellophane membrane, first against tap water and subsequently against distilled water until it no longer gives a test for ammonia with Nessler's reagent. At this time the sac should contain a copious precipitate of globulin.

Transfer the material to a 50 c.c. centrifuge tube. Centrifuge to spin down the globulin. Decant the supernatant liquid and wash the precipitate with distilled water.

Repeat the washing with distilled water twice. There is now a quantity of pure globulin, free from salts and ammonia.

Dissolve this material in normal saline and determine the globulin content by the Kjeldahl method.

Use dilutions of the material to set up the standard curve.

No exact procedure can be given for the preparation of this curve as modifications may be required depending upon the type of photoelectric colorimeter which is available. In general, however, the curve should be prepared in such a way that it will cover concentrations up to approximately twice the highest normal value to be expected in blood serum.

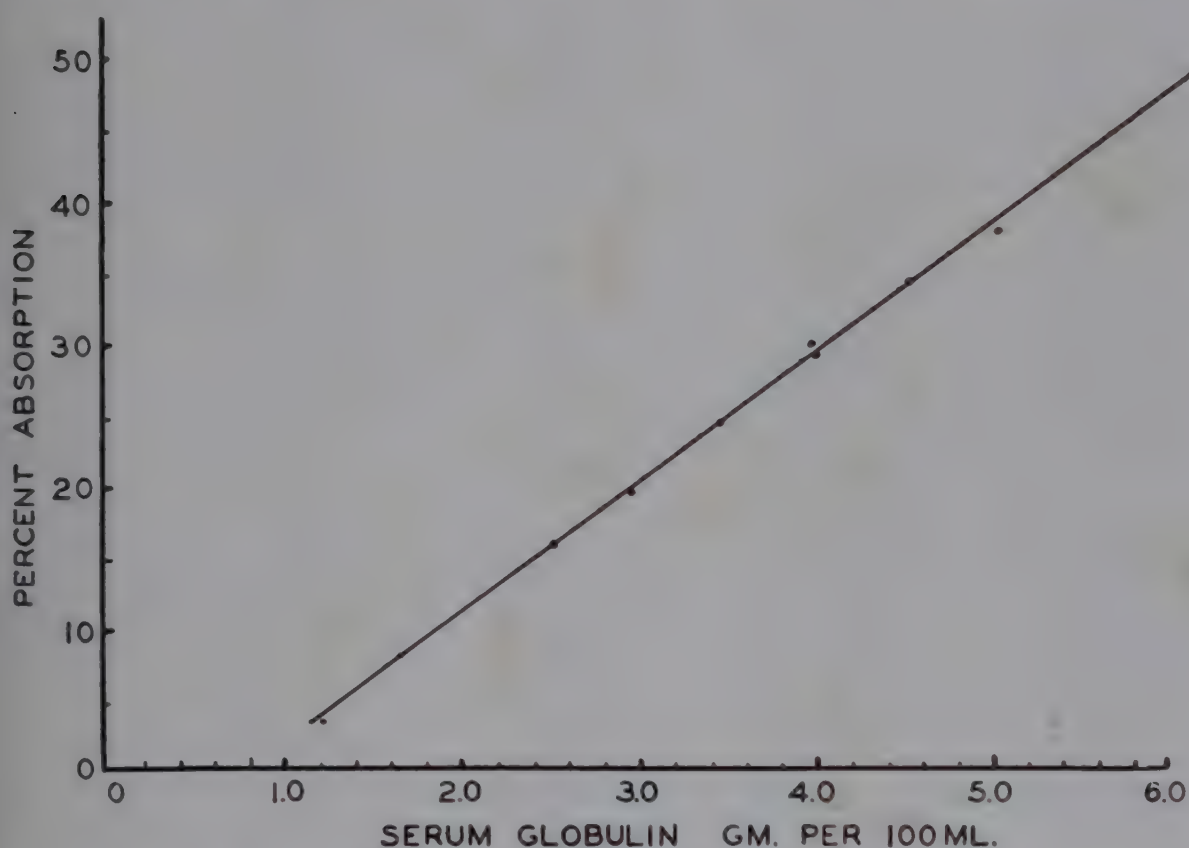


Fig. 115.—Example of a standard serum globulin curve.

Technic.—

(A) Globulin.—

Dilute 1 c.c. of blood serum to 10 c.c. with 1 per cent salt solution.

Mix 1 c.c. of this solution with 2 c.c. of 2 per cent gum ghatti.

Add 3 c.c. of saturated ammonium sulphate solution.

Mix thoroughly by gently inverting the tube several times.

Measure the turbidity in the photoelectric colorimeter, and estimate the globulin by comparison with a standard curve.

Vigorous shaking should be avoided in order to prevent the formation of innumerable fine bubbles in the solution and a persistent froth. It is necessary to wait for the complete disappearance of these fine bubbles before the turbid solutions are read, as otherwise too high values are obtained. A standard curve, such as shown in Fig. 115, must be prepared by treating different dilutions of a solution of serum globulin of known concentration. (See Serum Globulin Solution, under Reagents.)

Normal.—1.2 to 2.3 per cent.

²Looney, J. M.: Personal communication.

(B) Total Protein.—

Make a 1 per cent solution of serum by diluting the first serum mixture again in the proportion of 1:10 by the addition of 1 per cent salt solution.

To 2 c.c. of this dilution add 0.5 c.c. of 2 per cent gum ghatti and 2.5 c.c. of 5 per cent sulphosalicylic acid.

Read the resulting turbid solution in the colorimeter and determine the total protein by comparison with the standard curve for spinal fluid protein described by Looney and Walsh.¹ The value obtained from this curve times 100 gives directly the per cent of total protein. Obtain the albumin concentration by taking the difference between the total protein and the globulin values.

In sera containing large amounts of bilirubin there may be a precipitate when determining the globulin. If this occurs, the following procedure can be carried out.

In a 15 c.c. centrifuge tube, dilute one volume of serum with 2 volumes of physiologic saline and add 3 volumes of saturated ammonium sulphate solution.

Throw down the resulting precipitate in a centrifuge and remove the clear supernatant fluid.

Dilute 1 c.c. of this fluid to 20 volumes with 0.85% saline. Treat the resulting solution as for the total protein. The final reading multiplied by 1.2 will give the amount of albumin.

In less highly colored fluids a correction blank is run in which the sulphosalicylic acid or the ammonium sulphate is replaced by an equal volume of 0.85% saline solution, and the value obtained subtracted from the total value. This correction is very rarely needed for the total protein.

Turbidimetric readings may be made with white light or with a filter. As a rule the blue filter increases the sensitivity of the method and gives the best results.

If it is desired to avoid the preparation of a pure globulin solution, this can be accomplished by a slight modification of the method.

In this case, precipitate the globulin in a 10 per cent dilution of the original serum by adding an equal volume of saturated ammonium sulphate without the gum ghatti and separate the precipitate by centrifuging.

Dilute the supernatant liquid again 5 times and treat as above for total protein. For comparison, a new standard curve must be prepared from serum containing the same concentration of ammonium sulphate, as this high concentration of salt alters considerably the turbidity of the resulting solution.

Normal.—6.5 to 8.2 per cent.

Albumin and Globulin in Blood Serum, Method of Greenberg²

This method embodies essentially Howe's method³ of salting out the globulin with sodium sulphate solution. Since this sodium sulphate has almost no effect on the color developed, the albumin can be determined directly on an aliquot of the filtrate. This method affords, therefore, a satisfactory means for the analysis of the albumin and globulin, making use of 0.5 c.c. of serum.

Reagents.—**Sodium Sulphate Solution, 22.5%.—**

Place 22.5 gm. of sodium sulphate (anhydrous salt) in a 100 c.c. volumetric flask. Add sufficient hot water to dissolve, and dilute to the mark.

5N Sodium Hydroxide.**Standard Tyrosine Solution, 0.02%.—**

Place 0.2 gm. of tyrosine in a 1 liter volumetric flask. Dilute to the mark with N/10 hydrochloric acid.

¹Looney, J. M., and Walsh, A. I.: *J. Biol. Chem.* 127: 117, 1939.

²Greenberg, D. M.: *J. Biol. Chem.* 82: 545, 1929.

³Howe, P. E.: *J. Biol. Chem.* 49: 93, 1921.

Folin's Phenol Reagent (Folin and Ciocalteu).—

Place 100 gm. of sodium tungstate

25 gm. sodium molybdate

and 700 c.c. distilled water in a 1500 c.c. Florence flask connected by a ground glass joint to a reflux condenser.

Add 50 c.c. 85% phosphoric acid and
100 c.c. concentrated hydrochloric acid.

Reflux gently for ten hours. Cool.

Add 150 gm. lithium sulphate.

50 c.c. distilled water.

Few drops liquid bromine.

Boil fifteen minutes without a condenser to remove excess bromine. This should be carried out in a fume closet because of the poisonous nature of bromine fumes.

Cool. Transfer to a 1 liter volumetric flask.

Dilute to 1,000 c.c. and filter.

The reagent should be kept in a glass-stoppered, brown bottle and should be a golden yellow color. Reject it if it has a greenish tinge.

Equipment.—

- 2 medium size test tubes.
- 1 10 c.c. graduated pipette.
- 1 2 inch funnel.
- 3 50 c.c. volumetric flasks.
- 1 10 c.c. volumetric pipette.
- 2 2 c.c. volumetric pipettes.
- 1 5 c.c. volumetric pipette.
- 1 buret.
- Small filter paper.

Technic.—

Into a medium size test tube labeled "U," pipette 0.5 c.c. blood serum.

Add 9.5 c.c. of 22.5% sodium sulphate.

Agitate thoroughly.

Incubate in a warm air incubator for two hours at 37° C.

Filter through small filter paper, collecting all of the filtrate.

Label the tube containing the filtrate *Albumin*.

Wash tube "U" with two 3 c.c. washings using 22.5% sodium sulphate. Add the washings to the filter paper used for the original filtrate.

Wash the globulin precipitates on the filter paper twice more with 3 c.c. washings of sodium sulphate.

Discard all washings.

Globulin.—

Transfer funnel and filter paper to a 50 c.c. volumetric flask labeled "G."

Break filter paper and wash the precipitate into the flask with about 10 c.c. of 0.01 N sodium hydroxide.

Continue washing with distilled water until the flask is about half full.

Add 2 c.c. 5 N sodium hydroxide.

Add 3 c.c. phenol reagent from a buret, agitating the flask during the addition.

Dilute to 50 c.c. with distilled water.

Albumin.—

To a 50 c.c. volumetric flask labeled "A" pipette 5 c.c. of filtrate from the original collecting tube.

Add about 25 c.c. distilled water.

Add 2 c.c. 5 N sodium hydroxide.

Add 3 c.c. phenol reagent from a buret while agitating the flask.

Dilute to 50 c.c. with distilled water.

Standard.—

To a 50 c.c. volumetric flask labeled "S" pipette 4 c.c. standard tyrosine solution.
 Add about 25 c.c. distilled water.
 Add 2 c.c. 5 N sodium hydroxide.
 Add 3 c.c. phenol reagent from a buret while agitating the flask.
 Dilute to 50 c.c. with distilled water.
 Allow albumin, globulin, and standard to stand for 5 to 10 minutes and compare in a colorimeter.
 Set **unknown** at 10 and compare with the standard.

Total Serum Protein.—

To a 50 c.c. volumetric flask labeled "Total Protein" add 0.2 c.c. serum.
 Add about 25 c.c. distilled water.
 Add 2 c.c. 5 N sodium hydroxide.
 Add 3 c.c. phenol reagent from a buret while agitating the flask.
 Dilute to 50 c.c. with distilled water.
 Allow to stand for 5 to 10 minutes and compare with a standard prepared as for albumin and globulin.

Calculation.—

Set the **unknown** at 10 mm. on the colorimeter. Match with the standard and obtain the reading of the standard. Use the following formula to compute the result in any given case:

Reading of the standard $\times 0.664 = \% \text{ of albumin.}$

Reading of the standard $\times 0.288 = \% \text{ of globulin.}$

Reading of the standard $\times 0.8 = \% \text{ of total protein.}$

Total protein $\% - \text{albumin } \% = \text{globulin } \%.$

Normal Findings.—The normal protein content of serum is 6 to 8%; normal albumin content of serum is 3.5 to 5.6%; and the normal globulin content of serum is 1.5 to 3.2%.

Determination of Plasma Proteins, Andersch and Gibson¹

If an alkaline solution of the blood protein is heated for a short time, the chromogenic value, when treated with Folin-phenol reagent, is markedly intensified. Thus serum globulin gives half again as much color when so treated, and serum albumin, twice as much. A maximum chromogenic effect is produced by one-half hour of heating in a water bath, using 10 per cent sodium hydroxide as the protein solvent.

This procedure was introduced into the following adaptation of the colorimetric method of Wu,² Wu and Ling,³ and the late modification of Greenberg.⁴ Further simplifications of the technic of the colorimetric protein method have been introduced as a rapid and more accurate colorimetric procedure for the determination of blood proteins.

Greenberg, in his modification, used sodium sulphate according to the Howe separation of protein.⁵ The necessity of keeping the sodium sulphate at 37° C. to hold it in solution and the difficulty of filtration, besides the long time necessary for the complete precipitation of the globulin, induced Andersch and Gibson to discard the Howe procedure and use the precipitation with ammonium sulphate. Robertson⁶ reviewed the methods for the separa-

¹Andersch, M., and Gibson, R. G.: *J. Lab. & Clin. Med.* **18**: 816, 1933.

²Wu, H.: *J. Biol. Chem.* **51**: 33, 1922.

³Wu, H., and Ling, A.: *Chinese J. Physiol.* **1**: 161, 1927.

⁴Greenberg: *J. Biol. Chem.* **82**: 545, 1929.

⁵Howe: *J. Biol. Chem.* **49**: 109, 1921.

⁶Robertson, T. B.: *J. Biol. Chem.* **11**: 179, 1912.

tion of protein and concluded that the ammonium sulphate precipitation was the most satisfactory. Wu, and Wu and Ling, as well as Cullen and Van Slyke,⁷ also found this a satisfactory separation.

Wu had noted in his colorimetric determination of protein that if the protein precipitate were allowed to stand with sodium hydroxide for a short time, or with sodium carbonate for a long time, the chromogenic value was increased, but he made no attempt to incorporate this into his method.

It was found that by heating for one-half hour, the chromogenic value not only is constant and maximum, but also the tyrosine equivalents for the plasma proteins are roughly the same. This permits the determination of the total proteins of plasma and spinal fluid with sufficient clinical accuracy, irrespective of the protein partition.

The development of color with the Folin-phenol reagent⁸ is considered satisfactory. This reagent gives some precipitate; this, however, has no effect on the color, and settles so rapidly that it does not interfere with the colorimetric reading. The modified reagent by Folin and Ciocalteu⁹ gave an appreciably deeper color with the alkaline protein than did the original reagent as stated by Greenberg.

Reagents.—

Saturated Solution of Ammonium Sulphate.—

Dissolve 54 grams ammonium sulphate, c.p., in 100 c.c. distilled water.

20% Trichloroacetic Acid.—

Dissolve 20 grams of trichloroacetic acid in about 50 c.c. distilled water and dilute to 100 c.c. with distilled water.

10% Sodium Hydroxide.—

Standard Tyrosine Solution.—

20 mg. % solution. See page 304.

Phenol Reagent (Folin).—

See page 305.

Saturated Solution of Sodium Carbonate.—

Dissolve 22 grams of sodium carbonate, c. p., in about 80 c.c. distilled water using heat. Filter after diluting to 100 c.c. with distilled water.

Equipment.—

- 3 15 c.c. centrifuge tubes.
- 4 5 × 5/8 inch test tubes.
- 6 1 c.c. graduated pipettes.
- 3 2 c.c. volumetric pipettes.
- 2 3 c.c. volumetric pipette.
- 1 buret.

Technic.—

Into a 15 c.c. centrifuge tube pipette 1 c.c. blood plasma.

Add 2 c.c. distilled water.

Add 1 c.c. saturated ammonium sulphate.

Mix and centrifuge after 10 minutes.

Decant supernatant fluid for *albumin* and *globulin* and label tube "A."

Reserve the precipitate for *fibrinogen* and label tube "B."

To a 15 c.c. centrifuge tube pipette 1 c.c. of supernatant fluid from "A." Label tube

Albumin and Globulin.

⁷Cullen, G. E., and Van Slyke, D. D.: J. Biol. Chem. 41: 587, 1920.

⁸Folin, O., and Denis, W.: J. Biol. Chem. 22: 307, 1915.

⁹Folin, O., and Ciocalteu, V.: J. Biol. Chem. 73: 627, 1927.

Add 0.5 c.c. distilled water.

Add 1.0 c.c. saturated ammonium sulphate.

Incubate 12 hours at room temperature or 15 minutes at 40° C.

Centrifuge 5 to 10 minutes at 1000 r.p.m.

Decant supernatant fluid for albumin determination and label tube *Albumin*.

Add 5 c.c. distilled water to the sediment in the centrifuge tube and label tube *Globulin*.

Pipette 2 c.c. of supernatant fluid from *Albumin* tube to similarly labeled tube.

Add 3 c.c. distilled water.

Add 1 c.c. of 20% trichloroacetic acid to both *Albumin* and *Globulin* tubes.

Centrifuge both tubes 5 to 10 minutes at 1000 r.p.m.

Decant the supernatant fluid and retain the sediment.

To "B," albumin, and globulin sediments, add 0.5 c.c. of 10% sodium hydroxide.

Heat in a boiling water bath 30 minutes.

Add 7.5 c.c. distilled water to each tube.

Standard:

To a small test tube labeled "S" pipette 2 c.c. of standard tyrosine solution.

Add 6 c.c. distilled water.

To the three unknowns, that is "B," *Albumin*, and *Globulin*, and "S," add 1 c.c. phenol reagent, from a buret, while agitating the tubes.

Add 3 c.c. of saturated solution of sodium carbonate.

Invert tubes to mix and set aside for 30 minutes.

Compare in a colorimeter with the Standard set at 10.

Calculation.—The tyrosine equivalents are:

1 mg. tyrosine = 12.9 mg. albumin.

1 mg. tyrosine = 13.1 mg. globulin.

1 mg. tyrosine = 13.0 mg. fibrinogen.

Albumin.—

$$\frac{10}{R} \times \frac{0.4}{0.2} \times 100 \times 12.9 = \text{mg. albumin per 100 c.c.}$$

$$\text{or } \frac{25.8}{R} = \text{per cent albumin.}$$

Normal.—3.5 to 5.6%.

Globulin.—

$$\frac{10}{R} \times \frac{0.4}{0.25} \times 100 \times 13.1 = \text{mg. globulin per 100 c.c.}$$

$$\text{or } \frac{20.96}{R} = \text{per cent globulin.}$$

Normal.—1.3 to 3.2%.

Fibrinogen.—

$$\frac{10}{R} \times \frac{0.4}{1.0} \times 100 \times 13.0 = \text{mg. fibrinogen per 100 c.c.}$$

$$\text{or } \frac{5.2}{R} = \text{per cent fibrinogen.}$$

Normal.—0.2 to 0.4%.

Total Plasma Protein.—

Due to the close agreement of the tyrosine equivalents, it is possible to determine total protein by using 13 as the tyrosine equivalent.

Technic.—

Dilute plasma 1:10 with saline, transfer 1 c.c. to a 15 c.c. centrifuge tube, and add 4 c.c. of water and 1 c.c. of trichloroacetic acid. Centrifuge out the precipitate, and pour

off the supernatant fluid. Dissolve the precipitate in 0.5 c.c. of 10 per cent sodium hydroxide solution, and heat in a water bath for a half hour. The color is then developed as given previously for the fractions. (See preceding method.) **Set the standard at 10.**

Standard.—The same standard is used as is used for the fractions.

Calculation.—

$$\frac{10}{R} \times \frac{0.4}{0.1} \times 100 \times 13.0 = \text{mg. protein per 100 c.c.}$$

$$\text{or } \frac{52.0}{R} = \text{per cent protein.}$$

Normal.—6.2 to 8.4%.

Clinical Significance of the Serum Proteins Facts Relative to the Albumin-Globulin Ratio

The normal values for total proteins are 6 to 8 gm. per cent; for albumin, 3.5 to 5.6 gm. per cent; for globulin, 1.3 to 3.2 gm. per cent; the albumin-globulin ratio (A/G ratio), 1.5:1 to 3:1; the fibrinogen, 0.2 to 0.4 gm. per cent.

Albumin in kidney disease is derived from blood plasma and indicates increased permeability of the glomerular filter. Albumin, having a smaller molecule, is excreted in larger amounts than globulin or fibrinogen, markedly decreasing the albumin-globulin ratio in blood plasma when albuminuria is of severer grade.

Blood Proteins in Liver Disturbance.—It must be remembered that the liver is the source of serum proteins. Albumin is formed with greater difficulty than globulin in spite of the fact that it has a lower molecular weight. In cirrhosis of the liver, the total proteins may or may not be reduced, but there is an inversion of the albumin-globulin ratio. In ascites due to cirrhosis, there is a marked inversion of the albumin-globulin ratio, while little or no change in the albumin-globulin ratio occurs in ascites from other causes.

Albumin-Globulin Ratio in Various Diseases.—There are certain changes in albumin and globulin in various diseases. In **edema** of nephritic origin, the albumin is increased and the globulin is normal or decreased. In nutritional edema and that of beriberi, the albumin is decreased and the globulin is normal. In edema of cardiac origin, the albumin is normal or decreased, and the globulin is normal. In malnutrition, the albumin is decreased and the globulin is normal. In multiple myeloma, the albumin is normal or decreased and the globulin is increased. In nephrosis, the albumin is decreased and the globulin may be normal or increased. In pregnancy with toxemia, the albumin is decreased and the globulin is normal or increased.

Serum Proteins

In his discussion of this question, Kagan¹ states that the three components are distinct from chemical, physical, physiologic, immunologic, and clinical standpoints; therefore, their significance should be individually considered. The liver is the primary site of formation of serum albumin. Recent advances

¹Kagan, B. M.: South. M. J. 36: 234-238, 1943. (E. H. Sargent Co., Chicago, makes the Kagan instrument.)

indicate that the plasma cell and the reticuloendothelial system may be the sites of the origin of globulin. Albumin is of much greater importance than globulin in the maintenance of blood volume and fluid balance.

Globulin is more quickly regenerated following hemorrhage. Immunity is more dependent upon the globulin fraction-holding antibodies. Kagan points out that the A/G ratio (or albumin-globulin ratio) is misleading; what we really want to know is the absolute concentration of each.

Hypoproteinemia.—

Malnutrition is a common cause of hypoproteinemia. Again, even if properly absorbed, proteins may not be synthesized in liver disease. Abnormal metabolism of proteins is found in diabetes mellitus, pernicious anemia, and hyperthyroidism. Control of diabetes and treatment of pernicious anemia with liver extract are followed by rise in proteins. In hyperthyroidism, the protein rises to normal with proper surgery. Cancer of the stomach, liver, and pancreas shows a low protein. The lowest protein concentration is seen in nephrosis or the nephrotic stage of nephritis. There is a slight lowering of the protein in uncompensated cardiac lesions due to increased blood volume. Hypoproteinemia in pregnancy is due to improper dietary habits in addition to the protein drain of the fetus. Adequate diet or plasma injections cause the edema of pregnancy to subside.

Certain poisonings such as benzene, carbon tetrachloride, and phosgene cause hypoproteinemia, due to liver change. We recall a personal experience with a case of carbon tetrachloride poisoning at the Christian Hospital, St. Louis, where there were liver damage and hypoproteinemia, with recovery following the transfusion of plasma.

Carbon tetrachloride is toxic and occasionally fatal to man and experimental animals. Its lethal action is due to its damage to the kidney. Most of the cases reported are individual cases of poisoning, not necessarily industrial cases. Its excellent solvent properties, coupled with its noninflammability and inexpensiveness, have maintained the widespread use of carbon tetrachloride in industry and in the home. In passing, one must warn individual users of carbon tetrachloride for cleaning clothes and other objects to take every precaution to have plenty of ventilation in the room where this substance is used.

In the report of two cases, one fatal, Martin and others¹ give a very interesting review of the literature with tabulations of cases reported. The clinical picture is headache, nausea and vomiting, with occasional hematemesis, followed by mild icterus and later by oliguria, anuria, and retention of nitrogen. Pulmonary complications, such as edema and pneumonia, may occur as late as a week or more after exposure.

In the fatal case reported, the patient complained of pain in the vicinity of both kidneys and was passing grossly bloody urine. He had a history of daily exposure, over a period of six months, to carbon tetrachloride in a large, open vat. The patient also admitted the rather liberal use of alcohol. On the

¹Martin, W. B., Dyke, L. H., Jr., Coddington, F. L., and Snell, A. M.: *Ann. Int. Med.* 25: 488-497, Sept., 1946.

day before the onset of his symptoms, which happened to be rainy, he had shut himself in his car while he cleaned his uniform and the upholstery of the car with carbon tetrachloride.

The concentration of nonprotein nitrogen was 90 mg. per 100 c.c. of serum. Prothrombin time was 73 per cent of normal; the cephalin-cholesterol flocculation was reported to be weakly positive. Then followed jaundice, and the icteric index was recorded as 58 units. He appeared drowsy, vomiting persisted, and severe oliguria developed. Prothrombin time dropped to 50 per cent of normal, indicating progressively increasing hepatic damage. The nonprotein

TABLE 19.—DEATHS DUE TO CARBON TETRACHLORIDE AND REPORTED IN THE RECENT LITERATURE

REPORTED BY	CASES	DEATHS	CIRCUMSTANCES IN CASES IN WHICH DEATH OCCURRED
Allison, B. R. ¹	2	1	The patient was an alcoholic who drank an unknown quantity of carbon tetrachloride by mistake
Sanford, S. P. ²	1	1	A seaman used a bottle of carbon tetrachloride to clean his hands, forearms, and shoes, and died three hours later. Necropsy showed pulmonary edema
Konwaler, B. E., and Noyes, C. B., Jr. ³	3	1	Poisoning followed exposure in a closed compartment in which 1½ quarts of carbon tetrachloride had been volatilized; two other persons simultaneously exposed recovered (necropsy)
Sherman, S. R., and Binder, C. F. ⁴	4	1	The patient was cleaning a bomb sight in a room with poor ventilation and was overcome by fumes two or three times during the exposure (necropsy)
Forbes, J. R. ⁵	3	1	French seamen; exposure in a small, poorly ventilated compartment while cleaning clothes in fire extinguisher fluid
Willcutts, M. D. ⁶	3	2	All cases occurred on a submarine, following volatilization of carbon tetrachloride; fatality due to pulmonary edema nine days after exposure (necropsy)
Dillenberg, S. M., and Thompson, C. M. ⁷	20	1	
Eddy, J. H., Jr. ⁸	Unspecified	2	Ten patients, all ill enough to be hospitalized; exposure occurred in process of manufacture of a land mine; "a tremendous amount of the chemical was vaporized in a closed room having no mechanical ventilation"

¹Allison, B. R.: *Ann. Int. Med.* 16: 81-93, 1942.

²Sanford, S. P.: *U. S. Nav. M. Bull.* 41: 1486-1488, 1943.

³Konwaler, B. E., and Noyes, C. B., Jr.: *California & West. Med.* 61: 16-20, 1944.

⁴Sherman, S. R., and Binder, C. F.: *U. S. Nav. M. Bull.* 43: 590-599, 1944.

⁵Forbes, J. R.: *Lancet* 2: 590-592, 1944.

⁶Willcutts, M. D.: Quoted by Konwaler, B. E., and Noyes, C. B.³

⁷Dillenberg, S. M., and Thompson, C. M.: *Mil. Surg.* 97: 39-44, 1945.

⁸Eddy, J. H., Jr.: *J. A. M. A.* 128: 994-996, 1945.

nitrogen level rose to 174 mg. per 100 c.c. of serum. Patient's face became edematous. He became delirious, with convulsive seizures. There was clinical and roentgenologic evidence of bilateral pneumonia. He died the following afternoon. Autopsy showed enlarged kidneys. Differentiation between cortex and medullary portions of the kidney were indistinct. Microscopic examinations showed foci of necrosis in the liver. The hepatic cells in the centers of the affected portions showed advanced autolytic changes with complete loss of structure of many of the cells. Dilatation of the hepatic sinusoids, all of which were

TABLE 20.—LABORATORY DATA IN FATAL CASE OF CARBON TETRACHLORIDE POISONING

BLOOD CHEMICAL DATA										URINE			OTHER TESTS			
DATE, JULY	C.C.	I.I.	CO ₂	B.U.N.	N.P.N.	CL.	P. PROT.		CHOL.	BLOOD PRESSURE	WEIGHT, POUNDS	QUANTITY C.C.		U.N.	ALB.	CELLS
							TOTAL	ALBU- MIN								
5	3	25.4								125/ 80		None				Kahn negative Hgb. 12.5 gm. WBC 11,600 Segs. 69%; lymphs. 31%
6		30.8		62.5			4.7	3.3	140			525 Specific gravity 1.010		2	Loaded with RBC	Chest x-ray negative Platelets 202,860
8		21.7		70.3	91.5		5.1		155	160/100		2,105	5.1	Neg.	RBC 2-4 HPF	EKG normal Serum calcium 10.7 U.U. 1:80 Clotting time 3½ min.
11	1	11.6	64			396				190/120		2,580 Specific gravity 1.015		Neg.		WBC 8,050, Segs. 72% Erythrocyte sedimenta- tion rate 11 mm.
14				30.8		462	6.15	4.5		170/100	134½	2,800	6.5	Neg.	RBC None	Creatinine 2.1 Hgb. 14.5 gm. RBC 4,420,000
19		15.2		22.8	51.7					140/ 90	123	3,000		Neg.	RBC None	Urea clearance 48% Visual fields and fundi normal
23	Neg.	11.7		17.2	40.9		8.1	4.2		138/ 80	123	3,100		Neg.	RBC None	Platelets 320,000
25										125/ 80		2,900		Neg.	RBC None	Urea clearance 82% Sulphobromophthalein re- tension 45 min. 20% 60 min. 5%

Alb.—Qualitative urine test for protein.
Sugar was not present in any specimen examined.
B.U.N.—Blood urea nitrogen, expressed as milligrams per 100 c.c. of blood.
C.C.—24 hour cephalin-cholesterol flocculation test; graded 1-4.
Chol.—Blood cholesterol.
Cl.—Blood chloride expressed as NaCl.
Normal range 450-500.

Clotting time—Test tube method.
CO₂—Carbon dioxide combining power, c.c. per 100 c.c. of plasma.
EKG.—Electrocardiogram.
Hgb.—Hemoglobin (Haden-Hauser).
I.I.—Icteric index.
Lymphs.—Lymphocytes.
N.P.N.—Nonprotein nitrogen expressed as milligrams per 100 c.c. of serum.
Platelets—Method of Fonio.

P. Prot.—Plasma proteins.
RBC—Red blood cells.
Segs.—Segmented cells.
Serum calcium—Expressed as milligrams per cent.
Sedimentation rate—Cutler method.
U.N.—Urine urea nitrogen in grams per 24 hours.
U.U.—Urine urobilinogen in dilution (ratio).
WBC—White blood cells.

packed with red cells, was caused by the loss of supporting hepatic cells in affected regions. In the convoluted tubules and the loops of Henle there was marked destruction of the tubular epithelium. The cells were granular and many were completely degenerated. Numerous droplets of lipoid material were also demonstrable in the majority of the tubular cells on special staining.

In Table 20 are shown the laboratory data in this case.

One case of poisoning reported by Martin and associates occurred as a result of cleaning a uniform with a pint of carbon tetrachloride in an inadequately ventilated ship's compartment. Patient showed drowsiness, vomiting, and left subconjunctival hemorrhage. The liver was palpable. The upper part of the abdomen was diffusely tender. Patient was anuric for 24 hours; on the following day, urinalysis showed albumin and erythrocytes in quantity. By the second day he showed facial edema. Laboratory tests showed evidence of both hepatic damage and retention of nitrogen. Edema markedly increased and then rapidly subsided. This patient recovered.

Based primarily on the work of Minot and Cutler,⁹ who noted that low calcium levels increased susceptibility to carbon tetrachloride poisoning, calcium gluconate is frequently administered intravenously in treating acute carbon tetrachloride poisoning. In addition, the usual supportive measures, oxygen and intravenously administered fluids, should be employed. When anuria occurs, the physician faces the dilemma of the need for fluids and the patient's inability to excrete them. Confronted with this, Hagen and his associates¹⁰ employed diathermy over the renal region and felt that this was instrumental in the favorable outcome in their case. Although portal cirrhosis due to carbon tetrachloride has been described,¹¹ hepatic damage is usually a minor part of the problem with reference to human beings. The amino acid methionine and lipotropic substances, such as choline, are being given a clinical trial in mitigating the hepatic damage,¹² and appear to have some promise.

Hyperproteinemia.—

Both albumin reduction and globulin increase occur simultaneously in liver disease, cirrhosis, cancer, hemochromatosis, etc. Next to cirrhosis, acute glomerulonephritis is the commonest cause of such a change in both albumin and globulin.

Hyperproteinemia always means hyperglobulinemia. Since the reticulo-endothelial structures are supposed to be the site of origin of globulin, diseases involving this system cause hyperproteinemia. The highest values are seen in diseases of such structures, such as monocytic leukemia, multiple myeloma, and kala azar. The total protein concentration in multiple myeloma may be as high as 23.4 grams and in monocytic leukemia as high as 10.1 grams. Hence this determination is more important in multiple myeloma than is the Bence-Jones reaction—which may be negative.

⁹Minot, A. S., and Cutler, J. T.: *J. Clin. Invest.* 6: 369-400, 1928.

¹⁰Hagen, W. S., Alexander, H. A., and Prepard, T. A.: *Minnesota Med.* 23: 715-718, 1940.

¹¹Madding, G. F., and Butt, H. R.: *Proc. Staff Meet., Mayo Clin.* 13: 391-394, 1938.

¹²Beattie, J., Herbert, P. H., Wechtel, C., and Steele, C. W.: *Brit. M. J.* 1: 209-211, 1944.

Hyperproteinemia occurs in chronic infections, especially those of a suppurative nature; such as, lung abscess, bronchiectasis, tuberculosis, chronic osteomyelitis, subacute bacterial endocarditis, lupus erythematosus disseminata, and periarteritis nodosa.

What can we say of dehydrated states? Albumin is increased only temporarily in the early stage of dehydration, after which globulin is increased only temporarily. Kagan explains the edema of dehydration as follows: albumin is lost, dilution of the remaining albumin occurs when one injects saline or other fluids, so that there is insufficient osmotic pressure within the blood to prevent edema formation.

Hypoproteinemia

(1) Malnutrition

Dietary

- (a) Endemic and sporadic
- (b) Associated with chronic infection, pellagra, beriberi, etc.

Poor absorption

- (a) Idiopathic?
- (b) Diarrhea
- (c) Intestinal fistulae
- (d) Cancer of stomach, pancreas

Poor utilization?

- (a) Pernicious anemia, uncontrolled
- (b) Diabetes mellitus, unregulated

(2) Kidney Diseases

Nephroses—all types
Glomerular nephritis, chronic
Amyloid kidney

(3) Liver Diseases

Cirrhosis
Cancer

(4) Protein Dilution

Excess fluid administration

(5) Protein Loss

Hemorrhage, acute or chronic
Weeping wounds or skin lesions
Shock, surgical and traumatic

(6) Heart Failure

(7) Hyperthyroidism

(8) Chronic Poisoning

Benzene, carbon tetrachloride
War gas (phosgene)
Phosphorus

(9) Toxemias of Pregnancy

Hyperproteinemia

(1) Dehydration

Insufficient intake

Fluid loss

- (a) Intestinal obstruction and fistulae
- (b) Diarrhea, especially infants

- (c) Cholera
- (d) Diabetic acidosis
- (e) Vomiting
- (f) Burns
- (g) Heat cramps and heat exhaustion
- (h) Fulminant infections
- (i) Addison's disease

(2) Diseases Involving Reticuloendothelial System

Multiple myeloma
Monocytic leukemia

(3) Infections, Chronic

Suppurative
Ulcerative tuberculosis
Syphilis
Lymphopathia venereum
Subacute bacterial endocarditis
Periarteritis nodosa
Lupus erythematosus
Rheumatoid arthritis
Boeck's sarcoid
Malaria
Leprosy
Kala azar
Schistosomiasis
Filariasis
Trypanosomiasis

(4) Liver Disease

Cirrhosis of liver, early
Primary cancer of liver

Conditions Causing Hyperglobulinemia and Hypoalbuminemia Simultaneously

(1) Liver Diseases

- (A) Cirrhosis
 - (a) Primary
 - (b) Secondary to heart failure
- (B) Cancer
 - (a) Primary
 - (b) Metastatic

(2) Acute Glomerular Nephritis

(3) Combination of Two Diseases, the One Causing Increased Globulin, the Other Decreased Albumin, as

- (A) Increased globulin
 - (a) Syphilis
 - (b) Tuberculosis
 - (c) Lymphopathia venereum
 - (d) Periarteritis nodosa
 - (e) Subacute bacterial endocarditis
 - (f) Multiple myeloma

- (B) Decreased albumin
 (a) Nephritis
 (b) Malnutrition
 (c) Diarrhea

Amino Acid Nitrogen, Folin

Reagents.—

Stock Amino Acid Solution.—

Two stock solutions are prepared. The first is prepared by dissolving 268 mg. glycine (pure), and 1 gm. sodium benzoate in enough 0.07 N hydrochloric acid to make 500 c.c. The second is made by dissolving 526 mg. glutamic acid and 1 gm. sodium benzoate in enough 0.07 N hydrochloric acid to make 500 c.c.

Standard Amino Acid Solution (Containing 0.03 mg. nitrogen in 1 c.c.).—

Place 15 c.c. of the glycine stock solution and 15 c.c. of the glutamic acid stock solution in a 100 c.c. volumetric flask. Add 0.07 N hydrochloric acid containing 2 gm. sodium benzoate in 1,000 c.c. of the acid to the mark. This standard is used for the analysis of filtrates prepared from unclaked blood (see page 226).

Standard Amino Acid Solution (Containing 0.05 mg. nitrogen in 1 c.c.).—

Place 25 c.c. of the glycine stock solution and 25 c.c. of the glutamic acid solution in a 100 c.c. volumetric flask and add 0.07 N hydrochloric acid containing 2 gm. sodium benzoate in 1,000 c.c. of the acid to the mark.

Note.—The use of both glycine and glutamic acid in the standard is necessary because the shades of the color produced by the different amino acids vary somewhat.

Borax Solution.—

Dissolve 1.5 gm. of borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) in about 50 c.c. of distilled water in a 100 c.c. volumetric flask. Dilute to the 100 c.c. mark with distilled water.

Freshly Prepared 0.5% Sodium Beta-Naphthoquinonesulphonic Acid.—

Transfer 100 mg. of the quinone to a small flask. Add 20 c.c. distilled water and shake. Complete solution takes place rapidly.

Bleaching Solutions.—

Two bleaching solutions are necessary for the bleaching of the excess beta-naphthoquinonesulphonic acid reagent: (1) a 0.1 M solution of sodium thiosulphate and (2) an acid formaldehyde solution prepared by mixing 75 c.c. of 1.5 N hydrochloric acid and 25 c.c. of glacial acetic acid with 100 c.c. of 0.15 M formaldehyde. The 0.15 M formaldehyde may be made by diluting 11.3 c.c. of 40% formaldehyde to one liter.

Sulphate-Tungstate Solution.—

Dissolve 15 gm. of anhydrous sodium sulphate and 1.5 gm. of sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$) in enough distilled water to make 1,125 c.c.

Note.—This solution is to be added to the *standard* solution in the determination of amino acids when the *unclaked blood filtrate* is used because of the fact that the filtrate contains about 1.5% sodium sulphate and about 0.15% sodium tungstate which remains from the solutions used in the preparation of the filtrate.

Equipment.—

- 2 large test tubes (1 × 8 inches) graduated at 25 c.c.
- 1 10 c.c. volumetric pipette.

- 5 2 c.c. volumetric pipettes.
- 1 1 c.c. pipette graduated in 0.1 c.c.
- 1 5 c.c. volumetric pipette.

Technic.—

Pipette 10 c.c. of protein-free blood filtrate (Folin) (page 224) to a large test tube (1 × 8 inches) labeled "U" for unknown.

Pipette 1 c.c. of the standard solution (1 c.c. = 0.05 mg.) to a similar sized test tube labeled "S" for standard.

Add 2 c.c. of the 1.5% borax solution to "U."

Add 2 c.c. of the freshly prepared 0.5% beta-naphthoquinonesulphonic acid to each. Mix thoroughly.

Set both the "Unknown" and "Standard" tubes in a dark closet for from 18 to 24 hours.

After standing add 2 c.c. of the acid formaldehyde solution to each and 2 c.c. of the 0.1 M sodium thiosulphate solution to each.

Dilute "U" and "S" to 25 c.c. with distilled water.

Mix thoroughly and allow to stand for 4 or 5 minutes to allow for complete bleaching of the excess quinone reagent.

Read in a colorimeter.

Note.—If the unlaked blood filtrate (page 226) is used for the determination, 9 c.c. of the sulphate-tungstate solution is added to the standard to compensate for the sulphate-tungstate in the filtrate. The standard used is that containing 0.03 mg. nitrogen in each c.c. The procedure is the same as above.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 5 = \text{mg. amino acid nitrogen in 100 c.c. blood.}$$

If unlaked blood filtrate is used the calculation is

$$\frac{RS}{RU} \times 3 = \text{mg. amino acid nitrogen.}$$

Example.—Reading of standard is 20; the reading of the unknown is 30.

$$\frac{20}{30} \times 5 = 3.3 \text{ mg. amino acid nitrogen.}$$

or

$$\frac{20}{30} \times 3 = 2 \text{ mg. amino acid nitrogen.}$$

Normal is 2.3 to 3.74 mg. in 100 c.c. blood.

CLINICAL STUDY OF LIVER DISEASE

The clinical study of liver disease has in the past been dependent upon afforded a new method of investigation; the classification of the various forms mens in the living patient (see pages 1227 ff.) through needles or at operation has afforded a new method of investigation; the classification of the various forms of hepatitis into their phases has been the result of such a histological study.

We are indebted to Dr. Mitchell A. Spellberg, of Chicago, for permission to reprint from his excellent textbook, entitled *Diseases of the Liver* (published by Grune & Stratton, New York, 1954), the accompanying charts illustrative of liver tests and their clinical application.

SUMMARY OF LIVER TESTS AND THEIR CLINICAL USEFULNESS

General type	Test	Procedure	Normal Value	Abnormal Value	Clinical Significance and Usefulness
Pigment Metabolism	Bilirubin of serum	Total	1 mg.% or less	Above 1 mg.%	Hepatocellular damage, bile duct obstruction or increased production of bilirubin hemolysis
		1 min. prompt	0.25 mg.%	Above 0.25 mg.%	Hepatocellular damage or bile duct obstruction
		Delayed reacting	less than 1 mg.%	Above 1 mg.%	If prompt reacting is normal it indicates hemolysis
	Bilirubin of urine	Qualitative	o	+	Hepatocellular damage or bile duct obstruction
	Bilirubin clearance	1 mg. bilirubin per kg. I.V.	Under 5% at 4 hours	Over 5% retained	Sensitive test for hepatocellular damage—only in an icteric patient
	Urobilinogen of urine	Wallace & Diamond	+ 1:10 dil. or less	+ 1:20 dil. or more	
		2 hr. Watson	1 Ehrlich unit or less	Above 1 Ehrlich unit	Hepatocellular damage, or increased production of bilirubin = hemolysis
		24 hr. Quant.	0.2 mg. or less	over 0.2 mg.	
Excretory Function	B.S.P.	5 mg./kg. I.V. 45-min. sample	0 to 4% retention	Above 5%	Sensitive test of hepatocellular damage; positive in early mild portal cirrhosis, fatty liver and malignant metastasis to liver; limited usefulness in jaundiced patients
		10 cc. of 1% sol. I.V.	8 min. 55% 16 min. 35% or less retained	55%+ 35%+	Not as sensitive as the BSP test; dye is cheaper and less irritating
Conjugation and Detoxification	Hippuric acid synthesis	Oral 6 gm. sod. benzoate I.V. 1.77 gm. sod. benzoate	3 gm. or more in 4-hr. urine 0.7 gm. or more in 1 hr. urine	Below or above normal value	Indicates hepatocellular damage; limited usefulness in differential diagnosis of jaundice; I.V. test more accurate; renal dysfunction may invalidate test
	Para-amino hippuric (PAH) synthesis	3 gm. PAB orally; 1 hr. blood determination for PAH synthesis	100% synthesis	70% or less	Similar to hippuric acid test, but little data available
	Benzoyl glucuronate excretion	5.8 gm. Na benzoate orally—urine tested for glucuronate 2-4-6 hours	No glucuronate	Glucuronate excreted	Claimed to be more sensitive than hippuric acid test and helpful in differential diagnosis; chemical procedure is complicated
	Methylation of nicotinamide	Nicotinamide 50 mg. orally, 20 mg. I.V. Methylated comp. tested in 4-hr. urine	Much methylated comp. in urine	Methylation impaired	Index of hepatocellular damage, but not of clinical importance, because of complexity of chemical procedure

SUMMARY OF LIVER TESTS AND THEIR CLINICAL USEFULNESS (Continued)

Category	Test	Procedure	Normal Value	Abnormal Value	Clinical Significance and Usefulness
Carbohydrate Tests	Glucose tolerance	$\frac{1}{3}$ gm./kg. I.V.	Fasting blood sugar in 1 hour	Fasting blood sugar $1\frac{1}{2}$ to 2 hours	A useful physiological test to determine hepatocellular damage; may help in differentiation between hepatic and post-hepatic jaundice
	Adrenalin gluconeogenesis	Adrenalin 1:1000 to 0.01 cc. per kg. I.M.	Blood sugar rise 40 to 100 mg.% at 30 or 60 min.	Blood sugar rise less than 40 mg.%	Of special value in glycogen storage disease where no rise in blood sugar occurs; abnormal in other types of hepatocellular damage; about 60% correlation with other tests
	Insulin tolerance	0.1 u insulin per kg. I.V.	50% fall of blood sugar in 30 minutes; returns to normal in 90 to 120 min.	Hypoglycemia less marked; return to normal delayed	Test is of physiologic interest; indicates hepatic defect in COH metabolism, defective inactivation of insulin(?); impaired glycogenic effect of insulin(?)
	Galactose tolerance	Oral—40 gm. of galactose	4-hr. urine under 3 gm. galactose	Over 3 gm. galactose	Recommended for differential diagnosis of jaundice; galactose is expensive; test unreliable because of variability of intestinal absorption and renal excretion
		I.V. 0.5 gm./kg.	Under 20 mg.% galactose in blood at 75 min.	Over 20 mg. at 75 min.	Eliminates the disadvantages of the oral test; sensitive test of parenchymatous damage; negative in extrahepatic obstruction; value limited by technical complexity (fermentation)
	Lactate clearance Pyruvic acid	75 mg. sod. d-lactate/kg. I.V. Determination in blood	0 lactate after 30 min. in blood	Above 5 mg.% in blood Marked elevation in blood and spinal fluid	Of experimental rather than clinical interest Interesting from physiological point of view; especially elevated in hepatic coma; may be of prognostic value
Protein Metabolism	Gamma Globulin	Quantitative electrophoretic or salting out Turbidity $(\text{NH}_4)_2\text{SO}_4$ NaCl	0.7 gm.—1.3 gm.% or less 0 to 2 u	Above 1.3 gm.% 3 u or above	Very sensitive; shows an elevation in early and mild hepatocellular damage; very high in diffuse inflammation of liver, hepatitis, post-hepatic cirrhosis, cholangiolitic cirrhosis; increase not so marked in nutritional cirrhosis but may be elevated in other infectious processes and multiple myeloma
	ZnSO ₄ turbidity	Tests chiefly γ globulin concentration	4 to 12 u	Above 12 u	High in inflammatory disease of liver; inhibited by hyperbilirubinemia and lecithin

SUMMARY OF LIVER TESTS AND THEIR CLINICAL USEFULNESS (Continued)

General Type	Test	Procedure	Normal Value	Abnorml Value	Clinical Significance and Usefulness
Protein Metabolism (Continued)	Serum albumin	Electrophoretic salting out	3.2 to 4.4 gm.	Under 3.2 gm.	Not an early sign of hepatic dysfunction; decrease, especially when marked, is a sign of severe liver disease, especially prone to occur in advanced cirrhosis and hepatic necrosis; increase of the globulin/albumin ratio is a sign of severe hepatic disease
	Cephalin cholesterol flocculation		0 to 1+ in 48 hours	2+ to 4+ in 48 hours	Flocculation depends on increased globulin, and depression and qualitative alteration of albumin; sensitive test of active parenchymal disease; positive in similar conditions as the gamma globulin; may be the first positive test in hepatitis, but may be negative in inactive cirrhosis
	Thymol turbidity	30 min. turbidity with thymol reagent	0 to 5 u	Over 5 u	Positive test depends on elevation of gamma globulin, depression of albumin, lipid and lipoprotein elevation; highly positive in diffuse inflammatory diseases of liver: hepatitis, biliary and postnecrotic cirrhosis; inhibited by post-hepatic jaundice; may be negative or only mildly positive in hyperglobulinemias, such as multiple myeloma; may be slightly positive in nonhepatic inflammations
	Thymol flocculation	18 hr. flocculation	0 to 1+	2+ to 4+	Similar, but not identical with the thymol turbidity test; depends apparently on qualitative change in globulin; may remain positive in chronic hepatitis after the turbidity test has become negative

SUMMARY OF LIVER TESTS AND THEIR CLINICAL USEFULNESS (Continued)

Protein Metabolism (Continued)

Test	Procedure	Normal Value	Abnormal value	Clinical Significance and Usefulness
18-hour turbidity ratio	Turbidity 18 hrs. Turbidity 30 min.	85% or higher	Below 85%	Another way of expressing the thymol turbidity and flocculation; the greater the flocculation, the more positive the test becomes
Colloidal gold test		0 to 1+	2 to 4+ or 5+	Sensitive test of hepatocellular damage; depends on elevated globulin and decreased albumin; technically more complicated
Colloidal red	Flocculation of scarlet red	0 to 1+	2 to 4+ or 5+	Less sensitive than other flocculation tests, but gives fewer false positives; more likely to be positive in inflammatory diseases of liver
Amino acid tolerance test	40 cc. of 10% protein hydrolysate I.V.	Fasting level at 35 to 95 min.	Fasting level after 95 min.	More of experimental than clinical interest
Prothrombin time	Undiluted plasma	12 to 14 sec.	Above 15 sec.	Becomes positive in late and advanced liver disease; hence is more of prognostic than diagnostic importance
Vitamin K response	10 to 20 mg. I.V. or I.M.	10 + or more increase in prothrombin in 24 hrs.	No increase	In presence of jaundice and prolonged prothrombin time, valuable in differentiating between hepatic and post-hepatic causes of obstruction
Vitamin K tolerance test	76 mg. vit. K I.V. for 4 successive days	See page 32		Can be done even when prothrombin time is normal, and is reported as sensitive indicator of hepatic dysfunction; technically is cumbersome because of repeated determinations
Dicumarol response	100 mg. of dicumarol	No prothrombin fall	Fall to 60% of normal	A dangerous test should the prothrombin time get out of hand; dangerous to use dicumarol in patients with hepatic disease
Antithrombin	Plasma	20 to 25 sec.; 5 min. incubation	Below 20 sec.	Antithrombin depressed only in severe progressive hepatic necrosis and decompensation; insensitive test but important as prognostic sign

Lipid Metabolism

Lipids Lipid	Quantitative Turbidity	16 to 30 u	Above 30 u	Increased in extrahepatic obstruction, cholangiolitic cirrhosis and fatty liver; normal or decreased in other types of liver disease
Fat tolerance	100 gm. cotton-seed oil orally	Serum lipids 65% above fasting at 6 hrs. return to fasting level at 9 hrs.	6-hr. rise not so high; 9 hr. rise above fasting level	Defective fat metabolism in liver disease; not a practical test
Cholesterol	Total	120 to 300 mg.%	Increased Decreased	In extrahepatic obstruction, certain types of biliary cirrhosis In severe hepatocellular damage

SUMMARY OF LIVER TESTS AND THEIR CLINICAL USEFULNESS (Continued)

General Typ	Test	Procedure	Normal Value	Abnormal Value	Clinical Significance and Usefulness
Lipid Metabolism (Continued)	Cholesterol (Cont.)	Esters	65% or above of total	Decreased	The percentage of cholesterol esters is decreased early in hepatocellular damage; may decrease in post-hepatic jaundice because of hepatocellular damage; but in post-hepatic (obstructive) jaundice, the % decrease may be due to rise in free cholesterol; decrease of both total and esterified cholesterol is serious prognostic sign
Enzyme and Vitamin Tests	Vitamin A	Plasma level	40 to 100 micrograms	Decreased	Probably indicates defective absorption, although there is also defective storage in liver in hepatic injury; absorption is impaired in absence of bile in G.I. tract
		Tolerance 180,000 u orally	50% rise above fasting	No rise	
	Alkaline phosphatase	Bodansky	2 to 4	Increased	In post-hepatic jaundice, cholangiolitic hepatitis and biliary cirrhosis, metastatic neoplasm of liver; may be elevated in absence of jaundice and associated with hepatocellular regeneration
		King-Armstrong	3 to 13		
		Huggins	3 to 15		
		Gomori	2 to 4	Decreased	If decreased in spite of jaundice, or increasing jaundice, may be a sign of progressive liver failure and lack of regeneration
	Cholinesterase		0.68 to 1.37 u or 130 to 310 u depending on method	Decreased	Sensitive test of hepatocellular damage; remains normal in extrahepatic obstruction; hence, useful in differential diagnosis; may be a sign of impaired protein synthesis in liver

LIVER FUNCTIONAL TESTS

Liver functional tests are performed with the end in view of estimating the functional activity of the liver in both medical and surgical conditions. A normally acting liver before, during, and after an operative procedure upon the liver or gall bladder lightens the burden of the surgeon and tends to assure him of a favorable outcome. A multiplicity of tests and procedures has been offered in the past twenty-five years for the purpose of medical and surgical diagnoses.

As Mann¹ has well stated, the liver is a dynamic organ and the character of its function and the capacity for any particular function vary greatly within relatively short periods of time. It should be remembered, too, that there is a dissociation of function of the liver, so that injury may impair one function to a greater extent than other functions. Normal physiologic conditions may impair some of the hepatic functions; for instance, a fat diet may produce an increase in fat content of the liver and a decrease in some hepatic functions. There may be hepatic injury with restoration of hepatic tissues without apparent loss of hepatic function. Only a small amount of normal hepatic tissue may maintain normal hepatic activities under protection from functional stress. Therefore, the results of functional tests may vary in relation to tissues and even a slight variation in procedures, such as changes in diet and length of fast preceding the test, may alter the results of the test. One test may show a normal function, while another would indicate impairment of function. It must be emphasized that only a test which places considerable physiologic stress on the organ would indicate impairment of function even though the organ might be badly injured.

The most important **functions of the liver** are: *first*, the secretion of bile and *second*, hepatic activity in relation to foodstuffs. The rate of secretion of bile may be accelerated by physiologic stimulants, such as certain foods and notably the bile acids. It must be taken into account that hepatic activities in relation to the foodstuffs begin immediately after the ingestion of food. The liver undergoes a definite diurnal cycle in regard to size and weight which seems to depend on the food products reaching it and is due mainly to variations in its content of water and glycogen and possibly fat and protein. The major activities in relation to the utilization of food by the liver are storage of foodstuffs, manufacture of food materials, and regulation of the latter. Carbohydrate as glycogen and fat may be stored in the organ in relatively large amounts: up to 20 per cent of glycogen and 40 per cent of fat. Some protein may also be stored in the hepatic cells. Amino acids are deaminized by the liver, and the nitrogenous portion is converted into urea by the hepatic cells. The function of the liver in relation to the dextrose content is well known. The liver also is the site of origin for several important physiologic substances such as heparin, the plasma proteins, and some of the essential factors for the coagulation of the blood; namely, fibrinogen and prothrombin.

Hepatic activity protects the organism from some harmful substances which may originate within the body or reach the blood stream from the outside. Alkaloids such as strychnine and nicotine are destroyed in the liver.

¹Mann, Frank C.: J. A. M. A. 117: 1577, 1941.

Many substances are made innocuous by conjugation, which occurs in the liver. The liver also protects the body from toxic substances in other ways than by actual destruction, such as excretion in the bile, temporary delay in the hepatic cells with release in amounts too small to be injurious to other more vulnerable sites of action. These functions are known as detoxicating functions.

The liver must be studied in relation to the circulation and flow of blood, and the circulation in relation to hepatic restoration. Restoration of liver tissue after hepatic injury depends upon the portal blood flow. The intra-hepatic circulation of blood is a most interesting phenomenon because this organ possesses both an arterial and a venous supply of blood. The hepatic artery supplies arterial blood to the hepatic parenchyma through several routes: arterial terminals emptying directly into the sinusoids; arterial branches emptying into the radicles of the portal vein proximal to the sinusoids; and arteriovenous anastomosis between the two vessels proximal to the sinusoids. It is evident that the liver may act as a storage reservoir for blood. The work of Grindlay, Herrick, and Mann² showed that the liver is an important site for the storage of blood. These investigations showed that under certain conditions the liver may alternately store blood and discharge stored blood.

The factor of diet in relation to the liver should be considered from the point of view of the character of the food that will be most suitable to protect the organ from stress and the diet that will permit the liver to maintain its function most efficiently. The liver is best prepared to meet stress when its store of both carbohydrate and protein is ample. The impaired liver appears to function best on a high carbohydrate diet with sufficient protein obtained from simple foods to maintain the requirements of the body for this foodstuff as well as to supply the organ with sufficient amounts for the synthesis of the specific products it elaborates. A diet which tends to increase excessively the fat content of the liver is detrimental.

The relation of the liver to vitamin K must be taken into consideration in determining liver function and liver functional tests. Bile must be present in the gastrointestinal tract for the absorption of the vitamin contained in food or of vitamin concentrates. Feeding of bile or bile salts with diets containing vitamin K is sufficient to relieve the hypoprothrombinemia. Severe hepatic damage is usually associated with hypoprothrombinemia. In chloroform³ and carbon tetrachloride injury to the liver, the hypoprothrombinemia is not altered by administration of large amounts of vitamin K. A severely damaged liver is unable to maintain the prothrombin level of the blood even when vitamin K is available. The liver is responsible for the formation of prothrombin, and prothrombin formation may be prevented either by injury to the liver or by deficiency of vitamin K.

In relation to the liver and the endocrines, Mann believes that hepatic function may be altered either directly or indirectly by many of the hormones. He stated in a previous communication⁴ that hepatic activity can be changed

²Grindlay, J. H., Herrick, J. F., and Mann, F. C.: *Am. J. Physiol.* 132: 489, 1941.

³Smith, H. P., Warner, E. D., and Brinkhous, K. M.: *J. Exper. Med.* 66: 801, 1937.

Brinkhous, K. M., and Warner, E. D.: *Proc. Soc. Exper. Biol. & Med.* 44: 609, 1940.

Bollman, J. L., Butt, H. R., and Snell, A. M.: *J. A. M. A.* 115: 1087, 1940.

⁴Mann, F. C.: *Am. J. Digest. Dis. & Nutrition* 4: 355, 1937.

by hormones, first, by an effect on the rate of body mechanism, second, by an effect on the intrinsic mechanism of the liver, either for storage or manufacture of food, and by an effect on the amount of available food materials reaching the liver. It is evident that the functions of the liver may be altered by different hormones in each of the respects mentioned.

Anesthesia has an important effect on hepatic function. Chloroform produces injury to the liver. Experimental observations show that with pentobarbital sodium anesthesia, the duration of the anesthesia was definitely prolonged after partial removal of the liver and decreased as the organ was restored. Experimental observations on dogs show that during anaphylactic shock the liver becomes greatly congested and the blood leaving the liver possesses blood pressure lowering properties. This problem has been clarified by the demonstration that histamine or a histamine-like substance is present in the blood of the dog during anaphylactic shock. Experiments by Ojers, Holmes, and Dragstedt⁵ demonstrate that in the dog the histamine content of the liver is reduced during anaphylactic shock. Evidence to date suggests that much of the histamine liberated into the blood during anaphylaxis in the dog comes from the liver.

One of the earliest methods of estimation of liver function is a study of retention of bromsulphalein. This method is still reliable but must be supplemented by many other tests which have been introduced into the diagnostic field during the last few years. Much information is obtainable as to the state of the functional activity of the liver in cases of jaundice by determination of the value of the serum bilirubin, its daily variations, and a knowledge of the anatomic changes which these may represent. The bromsulphalein test is especially to be recommended in cases of diseases of the liver not associated with jaundice.

In studying liver functional tests, one must remember the **physiology of bile pigment formation**. Bile pigment is derived from hemoglobin released from the dying red cells, occurring in the liver or outside the liver. The reticulo-endothelial system is responsible for the extrahepatic biliary pigment manufacture. Bilirubin manufactured by the reticuloendothelial system is constantly circulating in the blood with an average percentage in the blood stream of 0.2 mg. per 100 c.c. This quantity may be elevated by reason of changes in the cells of the liver and in the patency of the biliary passages. The polygonal cells in the liver take bilirubin from the circulation and excrete it into the biliary passages down into the duodenum. Most of the bilirubin in the intestine is changed by intestinal bacteria to urobilinogen, which, in turn, is changed to urobilin. Some of it is reabsorbed into the portal blood where it is removed by the polygonal cells and goes out in bile once more.

Bilirubin increased in the blood to the level of 2 mg. per 100 c.c. means visible jaundice in the patient. This increased amount in the blood stream is due to excessive bilirubin plus the inability on the part of the liver to eliminate it. In febrile and toxic conditions and also in anoxemia, there is increased

⁵Ojers, Gaylor, Holes, C. A., and Dragstedt, C. A.: The Liver Histamine in Canine Anaphylaxis, read before the American Society for Pharmacology and Experimental Therapeutics, Chicago, April 15, 1941.

bilirubin production, together with a decreased capacity of the liver to excrete it. Any block in the biliary passages will produce a backward stagnation and accumulation of undue quantities of bilirubin in the blood stream.

To determine the degree of jaundice, one must estimate the amount of bilirubin in the blood. The direct and indirect van den Bergh tests are the most useful for this purpose. The icterus index will give us information as to the degree of the jaundice. The direct tests of urine and feces tell us just what amount is present in these two excretions.

Bilirubin is found in the blood stream in undue quantities (1) when it is produced in excess of the capacity to excrete it; (2) when it is found as result of injury to liver cells and biliary passages, as in hepatogenous jaundice; (3) in obstructive jaundice, with an influx of bilirubin into the blood stream as the result of obstruction of large bile passages.

Bilirubin that has been secreted by the liver and then reabsorbed gives a prompt positive *direct* van den Bergh reaction, but bilirubin that has never passed through the liver cells but has been taken directly into the blood stream gives an *indirect* reaction. The first type is readily excreted by the kidneys, whereas the second type does not pass the kidneys unless it appears in very high concentration in the blood. What we term "biphasic" reaction is seen when there are both types of bilirubin in the blood serum. A prompt direct van den Bergh is found in obstructive jaundice; a delayed direct in normal, or non-obstructive, or hemolytic jaundice; a biphasic in continuation of obstructive jaundice; an indirect in hemolytic jaundice.

The direct reaction is made on the whole serum and the indirect on the alcoholic extract separated from serum after it has been precipitated by alcohol and ammonium sulphate.

Prompt appearance of a purplish-red color reaching its height within thirty seconds indicates a prompt direct reaction, using the modification of Thannhauser and Anderson. The biphasic reaction is indicated by the appearance within thirty seconds of the red color which gradually increases in intensity to a violet. A delayed direct reaction is one that begins only after one minute, or longer, with development of the red color.

An excellent summary of laboratory aids in early recognition of liver disease is found in a monograph by Hoffbauer.¹ He emphasized the limitation of laboratory procedures in the early recognition of liver disease, but he feels that these may serve as valuable adjuncts to clinical experience. He emphasized the necessity of performing multiple tests on a single patient and gave the results of certain laboratory tests carried out in the University of Minnesota Hospitals on patients without obvious jaundice. The standards for these tests are: they must be simple to perform, they must entail no risk and little inconvenience to the patient, and they must be generally available.

Four laboratory tests are recommended because of the recognized limitations and variabilities of each:

1. Measurement of serum bilirubin level.
2. Detection of abnormalities of the serum proteins.

¹Hoffbauer, F. W.: *Minnesota Med.* 28: 903, Nov., 1945.

3. Detection of urobilinogen in the urine.
4. Measurement of bromsulphalein excretion.

The measurement of serum bilirubin is a method of detection of latent icterus. The normal individual seldom exceeds a level of 1.0 mg. per 100 c.c. for the total serum bilirubin. Modern laboratory methods permit precise determination of the serum bilirubin.

The method of Malloy and Evelyn is preferred since it permits quantitative estimation of the van den Bergh reaction. A knowledge of what portion of the total bilirubin reacts promptly to the diazo dye and what proportion is of the indirect reacting variety is of considerable value. A slight modification of the Malloy-Evelyn technic by Ducci and Watson permits the distinction of the two types of pigment in a quantitative manner.

Obtain a measurement of the prompt direct component by taking a reading one minute after the van den Bergh reagent is added to the serum sample.

Determine the total serum bilirubin, including both direct and indirect reacting fractions, after alcohol has been added to the serum and sufficient time (15 minutes) has elapsed to bring out the maximum color.

The level of the prompt direct bilirubin (1-minute reading) seldom exceeds 0.2 mg. per 100 c.c. in the normal patient. Figures in excess of this probably denote regurgitation of bilirubin that has been acted upon by the liver cells. In pure retention jaundice, as encountered in some hemolytic states, the value of the total bilirubin may be decidedly elevated but the 1-minute fraction scarcely altered. Such a finding immediately directs attention to the true nature of the underlying pathology.

Hoffbauer called further attention to the fact that the icteric index has definite value in the recognition of latent jaundice. Values for the icteric index greater than 8 to 10 units denote abnormal increase, but care must be taken not to confuse carotinemia with true increases of bilirubin. Carotin in the serum, by virtue of its yellow color, produces a false elevation of the icteric index, a disadvantage not found with the quantitative van den Bergh reaction. The substance can be readily detected, however, by simple extraction of the serum with petroleum ether. The presence of bilirubin in the urine is in all probability related to regurgitation jaundice.

Detection of Abnormalities of Serum Proteins.—This can be accomplished by two tests, the cephalin-cholesterol flocculation test of Hanger, and the thymol turbidity test. With respect to the Hanger flocculation test, meticulous preparation of the reagents must be carried out if false positive reactions are to be avoided. The thymol turbidity test is performed by adding a small amount of serum diluted with saline to a buffered solution of thymol. Resulting turbidity is compared with the standards used in the Kingsbury method of determining urine protein. Reading is made at the end of 30 minutes. Values are recorded in arbitrary units related to the degree of turbidity. Normal serum does not exceed a value of 4 and pathologic values may range as high as 30. Cases of extrahepatic biliary obstruction usually give negative values.

Detection of Urobilinogen in the Urine.—This is a valuable test because the demonstration of urobilinogen in abnormal amounts in the urine is an indication of liver cell dysfunction.

Urobilinogen is formed in the bowel. It results from the reduction of bilirubin by anaerobic bacteria in the colon. A portion of the urobilinogen so formed is reabsorbed into the portal circulation and carried to the liver. The exact fate of this chromogen is not known, but it would appear that the normal liver removes it. In the presence of liver disease large amounts of urobilinogen may appear in the urine. Even slight disturbances of liver function may result in the occurrence of urobilinogenuria.

Urobilinogen is a colorless substance and can be detected by the pink to red color that results when Ehrlich's reagent (paradimethyl-aminobenzaldehyde in hydrochloric acid) is added to the urine. The addition of sodium acetate intensifies the color in a true reaction and helps to eliminate false positive tests. Normal urine will seldom give a positive Ehrlich test.

Watson Test.—(See page 74.)

In order to make a quantitative determination, the method of Watson is recommended. Collect all the urine for 24 hours and extract an aliquot. The normal individual does not exceed 3 mg. per 24 hours by this method but pathologic values may range as high as 50 to 100 mg. The urine specimen is collected for a 2-hour period, between 2 P.M. and 4 P.M. because the excretion of urobilinogen is usually at its maximum in the afternoon. Express the value in terms of so-called Ehrlich units. One such unit is approximately equivalent to 1 mg. of urobilinogen. A value of greater than 1 or 1.2 Ehrlich units for the 2 to 4 P.M. period can be considered abnormal.

The Measurement of Bromsulphalein Excretion.—This test has been used for years. Its chief disadvantage is that it is of little value in the presence of regurgitation jaundice. The test should not be employed if the icteric index is greater than 20 or the serum bilirubin greater than 2.0 mg. per 100 c.c. The dosage of 5 mg. per kilogram of body weight is recommended and the clearance time taken as 45 minutes. The normal individual will show no retention of the dye in the blood stream after 45 minutes. Hoffbauer* believes that for clinical purposes the test is probably the most reliable laboratory aid available to measure liver function in the individual without jaundice.

His final word regarding all these laboratory tests is significant—"They do not make diagnoses. If properly employed, though, they can be extremely useful in detecting certain disorders early and in following their progression or regression."

Liver Death

Many patients with undeniable gall bladder disease, obstruction, etc., seem to be in perfect condition for operation, yet untoward results occur in their post-operative lives, results which puzzle and astonish the clinicians. Fatalities following biliary tract surgery are most unexpected. Experienced surgeons often deny their existence until catastrophes happen in their own practices.

The explanation, according to clinical and experimental work, lies in an underlying hepatic damage or dysfunction which becomes more pronounced when the strain of surgery is put upon it. "Damaged cells of the liver, failing

*Loc. cit.

in their function, release into the circulation some toxic substance which is excreted by the kidneys but which is so potent that the convoluted tubules, unprepared by nature for such a load, break under it."¹

Liver and liver-kidney deaths can be avoided by the surgeon who remembers that liver damage may exist in even most unlikely subjects, and by the use of functional tests to estimate preoperative risk; further, by avoiding anesthetic agents which are harmful to the kidneys; and by postoperative therapy which will improve liver function.

There are various types of hepatic tests which can be carried out in a hospital to avoid liver deaths. They are:

1. **Tests of Bile Metabolism.**—The value of the *icterus index test* is to demonstrate latent or subclinical jaundice and fluctuations in the intensity of the icterus which cannot be detected clinically. It is not a true test of liver function.

With the *van den Bergh test*, the *direct* method means that whole bile, which contains bile acids and cholesterin as well as bilirubin, has been regurgitated into the blood stream, and that the underlying pathologic process is either an obstruction of the bile ducts or rupture of canaliculi with necrosis of the liver cells. These are the only two conditions which permit bile to escape from the canaliculi into the blood stream. The *indirect* reaction indicates that the bilirubin of the plasma has not been regurgitated from the canaliculi into the blood but represents pigment which the liver has not been able to remove from the blood stream. The underlying pathologic process may, therefore, be assumed to be an overproduction of bilirubin or a depressed excretory function of the liver, or both.

The *urobilinogen test* is based on the idea that urobilin, produced in the intestinal tract, is converted to bilirubin in the liver, *completely* if liver function is normal, *incompletely* if it is not, in which case it comes over into the urine as urobilinogen. If no urobilinogen is present in the specimen, it may be assumed that biliary obstruction is complete.

2. **Tests of Excretory Function.**—The *bromsulphalein test*, in general, is important from a standpoint of low-grade retention; that is, 4 to 12 per cent. With such retention, liver function is low. This test is said to indicate obstruction before jaundice is clinically evident.

The *hippuric acid test* depends upon the fact that the synthesis of glycine is affected in certain types of liver damage and that the output of hippuric acid, which is correspondingly diminished because of the lack of glycine combined with benzoic acid, serves as an index to this damage.

3. **Tests of Hepatic Function.**—These tests are of various types; first, tests of carbohydrate function, of which the galactose tolerance test is a type. The galactose tolerance test and other carbohydrate tests are based on the principle that in cases of hepatic dysfunction, the rate of utilization of a predetermined amount of carbohydrate will be less than in normal persons, and that therefore a greater amount of the substance will persist in the blood stream and will be excreted in the urine. It is believed that these methods are somewhat unreliable in that they affect only the reserve capacity of the

¹Boyce, F. F.: *Role of Liver in Surgery*, Springfield, Ill., 1941, Charles C Thomas.

organ. *The galactose test* means that a 40 gm. oral dose of galactose can be assimilated by a normal individual without the loss of more than 2.5 to 3 gm. of sugar in the urine in the five hours following its administration. But where there is hepatic injury, conversion of the galactose into glycogen is so seriously affected as to produce a degree of galactosuria above this level.

Tests for Liver Function

The Direct van den Bergh Reaction, Modification of Thannhauser and Anderson¹

The direct reaction is made on the whole serum and the indirect on the alcoholic extract separated from serum after it has been precipitated by alcohol and ammonium sulphate. Oxalated plasma or coagulated blood may be used but there must be clear serum with no hemolysis and the test must be performed as soon as possible after the withdrawal of the blood.

Reagents.—

1. **Sulphanilic Acid in Hydrochloric Acid.** This is made by dissolving 5 gm. sulphanilic acid in 50 c.c. of concentrated hydrochloric. Dilute to one liter with distilled water.

2. **Caffeine-Sodium Salicylate Mixture.** Caffeine 0.976 gm. and sodium salicylate 0.640 gm. are dissolved in a small amount of water and evaporated to dryness on the water bath. Dry this in the desiccator. Reduce to a powder and store in a bottle.

3. **Sodium Nitrite.** Dissolve 0.5 gm. sodium nitrite in 100 c.c. of distilled water.

When ready to make the test, mix 25 c.c. of the sulphanilic acid solution with 0.75 c.c. of the sodium nitrite. This is called the **diazo reagent**.

Test is made by placing 0.25 c.c. of clear serum or plasma, free from hemoglobin, in three small test tubes. To the first tube add 0.2 c.c. of water. To the second tube add 0.2 c.c. of freshly mixed diazo reagent, shake, and wait ten minutes. If any color appears in the second tube add 0.2 c.c. of diazo reagent to the third tube and note when the color first appears. This color development can be hastened by adding a small crystal of the caffeine-sodium-salicylate mixture described above. This third tube acts as a maximal control and the first tube as a negative control.

The prompt appearance of a purplish-red color reaching its height within thirty seconds indicates a prompt direct reaction. A biphasic reaction is indicated by the appearance within thirty seconds of the red color which gradually increases in intensity to a violet. A delayed direct reaction is one that begins only after one minute, or longer, with development of the red color. For further interpretation of this test see pages 326 and 329.

The Indirect van den Bergh Reaction

This is a colorimetric determination using a standard cobalt solution equivalent to 1 c.c. of bilirubin.

Reagents.—

1. **Sulphanilic Acid Mixture** as described under direct reaction.

2. **Caffeine-Sodium Salicylate Mixture**, as described under direct reaction.

3. **Sodium Nitrite**, as described under direct reaction.

4. **95% Ethyl Alcohol.**

5. **Saturated Solution of Ammonium Sulphate.**

6. **Standard Cobalt Solution.**—Dissolve 2.161 gm. of anhydrous cobalt sulphate in distilled water in a 100 c.c. volumetric flask and dilute to 100 c.c. with distilled water. This solution is subject to change. See sources of errors below.

¹Thannhauser, J. S., and Anderson, E.: *Deutsch. Arch. f. klin. Med.* 137: 179, 1921; *abst. J. A. M. A.* 77: 1292, October, 1921. Greene, C. H., Snell, A. M., and Walters, Waltman: *Arch. Int. Med.* 36: 248-272, 1925.

Technic.—

Place 1 c.c. of serum or plasma in a 15 c.c. centrifuge tube and add 0.5 c.c. of diazo reagent, mix, and allow to stand for two minutes. Now add 2.5 c.c. of 95% alcohol and shake. Add 1 c.c. of saturated ammonium sulphate solution. Shake the centrifuge tube thoroughly and centrifuge for five minutes. The tube now shows three layers, a clear layer at the bottom of ammonium sulphate, a middle layer with albuminous precipitate, and an upper layer of alcohol bearing the color. Remove this upper layer with a pipette. Compare this against the standard to determine the amount.

Use the standard cobalt solution, page 330. This is considered to be equivalent to 1 c.c. of bilirubin.

The calculation with colorimeter reading is as follows:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 4 \times 0.5 = \text{mg. of bilirubin per 100 c.c.}$$

Normal serum contains from 0.1 to 0.3 mg. per 100 c.c.

For interpretation of this reaction see pages 326 and 329.

Sources of Errors in Performance of Test.—

1. The standard becomes paler when exposed to light. It can be made stable by the addition of 2 drops of concentrated sulphuric acid to 500 c.c. standard solution, and should be kept in the dark.

2. The serum must be clear. The serum is cloudy during the absorptive periods of digestion. Serum usually becomes opaque on standing more than 24 hours in contact with the cells and must not be allowed to stand.

3. The slightest trace of hemolysis vitiates the result.

4. Substances other than bilirubin deepen the color of serum, such as carotin and xanthophyll. Several hours after the ingestion of food no effect upon the serum may be detected, but one cannot be certain as the power of absorbing coloring matter may vary in different individuals. The specimen should be obtained during fasting to rule out possible interference from such sources.

The Killian disc standard may be used in place of the standard cobalt solution. Unscrew the left-hand plunger of the Klett colorimeter and insert standard on top of the plunger.

Interpretation of the van den Bergh test is given on pages 326 and 329.

Urobilinogen Test**Method of Wallace and Diamond****Ehrlich's Aldehyde Reagent.—**

Mix 20 c.c. concentrated hydrochloric acid with 80 c.c. distilled water. Add 2 grams paradimethylamidobenzaldehyde. Stir until dissolved.

Technic.—

Add 1 c.c. of Ehrlich's aldehyde reagent to 10 c.c. of urine.

Warm the test tube.

A rose color appears if urobilinogen is present. The amount may be determined by diluting the specimen and observing the dilution at which the reaction is still present.

Quantitative Test for Urobilinogen, Watson

(See page 74.)

Methylene Blue Test for Bilirubin in Urine

In February, 1933, Fellingner and Menkes published their article on the "Quantitative Bilirubin Determination in the Urine With Methylene Blue Method."¹ Franke had previously suggested the use of methylene blue for

¹Fellinger, K., and Menkes, K.: Wien. klin. Wchnschr. 46: 133, Feb. 3, 1933.

the quantitative determination of bilirubin in the urine by determining the number of drops of methylene blue required to change a positive green color to a definite dark blue color. Twenty drops of a 2 per cent solution of methylene blue represented roughly one milligram of bilirubin present in the urine. In low concentrations the change from green to blue is definite. With greater concentrations of bilirubin in the urine, the change is not so clear-cut and the accuracy of the results is impaired by the increased volume of methylene blue in the test.

A standard containing a known amount of bilirubin was later developed. A definite bluish-green hue is obtained by mixing a solution of 1 c.c. of a 0.2 per cent methylene blue and 5 c.c. of alkaline fluid containing 1.0 mg. of bilirubin. This bluish-green hue is then matched by preparing a solution of alcoholic methylene blue in a weak potassium dichromate solution which is kept in an airtight container in a dark room. The test is then carried out by adding drop by drop a 0.2 per cent methylene blue solution to 5 c.c. of urine until it matches the prepared standard.

Myers,* in 1945, stated that it is not definitely known whether the methylene blue's changing to green in the presence of bilirubin is the result of a chemical reaction or due to a mixture of pigments. The latter seems to be more probable. The presence of the urobilin pigment normally found in urine will not give a positive test even though the urine be very dark and concentrated. The only known false positive results encountered in these tests have been found in urine containing blood and in several cases where there was an elevated temperature above 101° F. The possibility that the hemosiderin pigment of the blood may play a vital part in this test is likely. Two drops of blood hemolyzed in 10 c.c. of water will give a slightly positive test when methylene blue is added and if the number of drops of blood is increased, the green color is intensified. Istizin is the only drug that will give a false positive.

Technic.—

A test for occult blood must be made on the urine before making the methylene blue test, because if occult blood is present a false positive methylene blue test results.

To keep all factors as constant as possible, the size of the tubes, the volume of the test, and the number of drops of methylene blue for each test must never vary. Use tubes 16 by 150 mm.

(a) Qualitative Test.—

Place 10 c.c. of urine in the test tube and add 2 drops of Loeffler's methylene blue. Refer to Chapter X, Vol. II.

The solution remains dark blue if negative, but turns a brilliant green if positive. The reaction is immediate and is not altered by temperature or by the acidity or alkalinity of the urine. The difference in color between a negative and a positive test is such that the reading of the result is easy in both natural or artificial light.

(b) Quantitative Test.—

If the qualitative test is positive, it is well to make a quantitative determination as a routine procedure.

Use ten test tubes. Add urine as follows: 10 c.c. in the first tube, 9 c.c. in the second, then 8 c.c., 7 c.c., down to 1 c.c. in the tenth tube. A control may be run by using 10 c.c. of water without urine.

Add to the second tube 1 c.c. distilled water, 2 c.c. to the third, 3 c.c. to the fourth, until the volume in each tube is 10 c.c.

*Myers, C. P.: *J. Indust. Hyg. & Toxicol.* 27: 2, 52-55, Feb., 1945.

Add 2 drops of Loeffler's methylene blue to each tube and mix.

Record the last dilution of urine in which a positive reaction occurred. The control tube should give a negative reaction.

Value of Test*.—

1. By using methylene blue to test for bilirubin in the urine, positive results are obtained before the serum bilirubin becomes elevated and in most instances before the patient recognizes any of the symptoms of liver damage.

2. Bilirubin is eliminated in the urine in mild concentration as a "temporary spilling-over" of a renal threshold without elevation of the serum bilirubin as demonstrated by positive methylene blue test. This stage may be followed by the urine's becoming free of bilirubin, or it may progress to the stage of elevated serum bilirubin.

3. Following an elevation of serum bilirubin the urine becomes negative before the serum bilirubin returns to within normal limits.

It is realized that far more information is necessary before the full significance and accurate evaluation of the results of the methylene blue test can be fully established.

Modification of Harrison Test for Bilirubin in the Urine

Bilirubin may appear in the urine in cases of experimentally induced hepatitis before there is any elevation in the total serum bilirubin. Accordingly, there is need for a rapid test of the urine to detect the presence of bilirubin. Harrison¹ suggested a method which has been named for him. In this test barium chloride solution and urine are mixed in equal amounts, precipitate caught on filter, then treated with Fouchet's reagent which gives a green color if bilirubin is present. Hawkinson, Watson, and Turner² described a modification better adapted for usage in large numbers of tests. They stated it is equivalent to the original method of Harrison.

Reagents.—

Fouchet's Reagent.—

Dissolve 0.9 gm. of ferric chloride in enough 25 per cent trichloroacetic acid to make 100 c.c. solution.

Barium Impregnated Strips or Cotton Swabs.—

Allow pieces of extra thick and retentive filter paper (Schleich and Schull number 470) to remain briefly in a saturated aqueous solution of barium chloride. Allow them to dry in the air or, preferably, in a drying oven, then cut them into strips 4 inches long by $\frac{1}{2}$ inch wide. Use a single strip for one test, as described below. If a suitable type of thick filter paper is not available, cotton swabs may be used, although the resulting test is not quite as sensitive and clear-cut as with the filter strips. Prepare the swabs on ordinary applicator sticks, the cotton having dimensions of about $1\frac{1}{2}$ inches by $\frac{1}{2}$ inch. Dip these into the saturated aqueous solution of barium chloride; after drying, they are ready for use.

Technic.—

Place one end of a barium chloride impregnated thick filter strip in the urine sample to be tested, the strip being in an approximately vertical position, at least one-half extending above the surface of the sample. Allow the strip to stand in the urine for from thirty seconds

*Myers: Loc. cit., p. 332.

¹Harrison, G. A.: *Chemical Methods in Clinical Medicine*, London, 1937, J. & A. Churchill.

²Hawkinson, V., Watson, C. J., and Turner, R. H.: *J. A. M. A.* 129: 514-515, 1945.

up to two minutes, then withdraw it and place it on a piece of dry paper such as a paper towel or any other absorbent paper. Inspection of the filter strip will usually reveal somewhat more color in that area which corresponded to the surface of the urine. Drop 2 or 3 drops of Fouchet's reagent directly on this area. A positive test is denoted by the appearance of a green color, varying in intensity with the amount of bilirubin present. With smaller amounts the color is often detected as a faint green line running across the strip.

When the cotton swab is used, the method is varied slightly. Immerse the swab in the urine to be tested. Express the excess urine from the swab by rolling it against the side of the container. Drop 2 or 3 drops of Fouchet's reagent in the middle of the swab. A positive test is denoted as just described. The weaker reactions are less easy to interpret, in that the reaction is over a large area and hence more diffuse and not as clear-cut as the line reaction observed with the filter strip method. Nevertheless the swab method has considerable usefulness when the proper type of filter paper is not available. To date the only paper that has been found suitable has been the S and S number 470, but it is quite possible that other varieties which have not as yet been tried may prove just as satisfactory.

In a number of instances a series of dilutions of bilirubin-containing urines has been made to a point where the ordinary Harrison test was either questionable or absent. In each instance the results obtained with the present method were entirely comparable. Thus it is believed that the present filter strip method is at least equivalent to the original Harrison procedure.

It may be noted that barium has a peculiar virtue, possibly of catalytic nature, in this reaction. The test is not as sensitive when calcium chloride is used, although calcium salts appear to adsorb bilirubin from the urine just as efficiently as barium.

In connection with this test, Stokes and Osterberg³ reported a study with three objectives: (1) to determine whether any correlation existed between the degree of hyperbilirubinemia and the positivity of the methylene blue test; (2) to attempt to find, if possible, at what concentration of bilirubin in the urine the result of the test became positive; and (3) to demonstrate the nature of the test; that is, whether the color which is produced represents a chemical reaction or merely a blend of colors.

Twenty-eight patients who had diseases involving the liver and the common bile duct were studied. In all cases there was hyperbilirubinemia, and in twenty-two cases jaundice was present. There was not perfect correlation between the degree of hyperbilirubinemia and the positivity of the test.

Spectrotransmittance curves of urines giving positive tests and other solutions used in these experiments were determined by means of a Coleman Junior Spectrophotometer, Model 6. Urines giving a positive test had a spectrotransmittance characteristic of bilirubin with maximal absorption between 425 and 450 millimicrons. They found two absorption bands, one at 425 to 450 and one at 660 millimicrons, showing that the resulting green color is merely a blend of the original yellow and blue. That there is no new substance formed is evidenced by the lack of any new absorption band.

Their conclusions were:

1. They did not observe perfect correlation between the concentration of bilirubin in the blood serum and the degree of positivity of the methylene blue test.

2. The methylene blue test performed on urine with artificially produced bilirubinuria first became positive at a concentration of 2.0 mg. of bilirubin per 100 c.c. of urine.

³Stokes, G. D., and Osterberg, A. E.: *Proc. Staff Meet., Mayo Clin.* 21: 14, July 10, 1946.

3. Under the conditions of their experiments it would appear that the color which results when methylene blue is added to urine which contains bilirubin is due to a blend of blue and yellow colors rather than to some specific chemical reaction between methylene blue and bilirubin.

LaMotte Blood Bilirubin Outfit

This set is used for the quantitative determination of bile pigments in the blood—the direct and indirect van den Bergh reaction. Serum is treated with a diazotizing reagent and the red color which results from its reaction with bilirubin is taken as an index of the type and extent of bilirubinemia, depending on the rate of appearance and depth of color. Comparison is made with permanent color standards.

Icterus Index

Icterus index is a test for measuring the intensity of the yellow color of the serum. This color depends upon the content of bilirubin. The presence of other coloring matters, however, constitutes serious sources of error. These may be hemoglobin, hematin, carotin (from ingestion of carrots), and other lipochromes. In order to avoid most of these sources of error, blood should be taken from the patient in a fasting condition. The serum must be clear and without hemolysis. It is not primarily a test for determining jaundice, rather it is useful in following the course of the case from day to day.

Interpretation of Icterus Index.—The icterus index normally ranges from 4 to 6. Figures of from 4 to 6 are obtained when there is no bile in the urine and no jaundice of any kind. Figures of from 6 to 15 are obtained in cases of latent jaundice, but even at this time there may be no bile in the urine and very little staining of tissues. When the figure is over 15, clinical icterus is apparent, at which time we find bile in urine and staining of the tissues. Increases in icterus index are seen in hemolytic infections, pernicious anemia, and biliary obstructions. In all cases, carotinemia must be excluded. There is a low index after hemorrhage.

Reagents.—

Potassium Dichromate Standard Solution

Potassium dichromate, reagent grade	50 mg.
Sulphuric acid	0.2 c.c.
Distilled water	500 c.c.

Dissolve the dichromate in 400 c.c. of the water, add the acid, then the remainder of the water. Keep this reagent in a dark colored glass-stoppered bottle.

Physiologic Saline Solution (0.9%) (see Vol. II, Serology, Chapter XIII)

Technic.—

Withdraw blood with a dry needle and syringe into a dry centrifuge tube and centrifuge to obtain a clear serum.

Place the serum in a colorimeter cup and match against the potassium dichromate solution.

If the color of the serum is deeper than that of the standard, dilute 1 c.c. of the serum with 0.9% sodium chloride solution in a graduated cylinder until it is lighter than the standard. Record the dilution. Compare the serum and the standard in a Klett or Duboseq type colorimeter.

Calculation:

$$\frac{\text{Reading of the Standard}}{\text{Reading of the Serum}} \times \text{dilution} = \text{icterus index.}$$

Example:

$$\frac{15}{12} \times 20 = 25, \text{ the icterus index.}$$

The normal is 4 to 6.

Icterus Index Using Hemolyzed Serum, Method of Dingee***Reagents.—**

Standard Solution of Potassium Dichromate. (See preceding technic.)

70% Acetone in Physiologic Saline.—Dilute 70 c.c. of acetone to 100 c.c. with 0.85% saline.

Technic.—

Dilute 1 c.c. of blood serum with 70% acetone in saline to 12 c.c.

Filter and collect all of the filtrate.

Compare in the colorimeter.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 12 = \text{Icterus index.}$$

LaMotte-Pigford Icterus Index Comparator¹

This is a test to determine the icterus index as an aid in detecting conditions of latent or obstructive jaundice, anemias, malignancies, and liver damage in arsphenamine treatment. From 3 to 5 c.c. of blood are allowed to clot, the clear serum is placed in a test tube, and compared with standard color tubes of known indexes. The use of a series of color standards permits comparisons to be made over several parts of the scale by the simple procedure of diluting the serum to varying degrees.

Tests of Excretory Function of the Liver**Rose Bengal Test**

This test was developed by Delprat, Stowe, and Weeks.²

Technic.—

Inject 5 to 10 c.c. of 1 to 2 per cent solution of rose bengal in physiologic saline solution with careful precautions into the vein at the bend of the elbow. Wait two minutes and then withdraw 8 c.c. of blood into a bottle prepared for oxalation of blood, such as is recommended for blood chemical examinations. The plasma of this sample is used as a standard. Exactly six minutes later, withdraw another 8 c.c. of blood and use this as a test sample. Normally 50% or less of the injected dye should be present in the blood eight minutes after the injection has been made.

Centrifugalize both specimens. Take 3 c.c. of plasma from each sample, add 6 c.c. of acetone to precipitate the proteins, and recentrifugalize. If it is noted that the plasma is yellow from the presence of bile pigment, add 3 drops of saturated solution of sodium hydroxide and let stand for fifteen minutes. Centrifugalize again and compare with the standard in a colorimeter. Since the normal is only 50 per cent of the standard, higher values than this mean disturbance or lowered liver function. The dye has a photosensitizing effect so that the specimen should be kept in the dark and the patient should be protected from direct sunlight for some hours after the use of the dye.

*Dingee, J. L.: Lab. Digest 6: No. 2, 1942.

¹Pigford, R. G.: J. Lab. & Clin. Med. 13: No. 7, 1928.

²Delprat, G. D.: Arch. Int. Med. 32: 401-410, 1923. Stowe, W. P., Delprat, G. D., and Weeks, Alanson: Am. J. Clin. Path. 8: 55-60, 1933.

Calculation is as follows:

$$200 - \frac{200 \text{ RS}}{\text{RU}} = \text{per cent of normal liver function.}$$

RS is the colorimeter reading of the first, 2-minute, sample.

RU is reading of the second, 8-minute, sample.

It must be remembered that there are difficulties about the performance of this test, namely, the obtaining of a satisfactory standard, the adequate mixing of the dye in the blood stream, and the rigid time requirements of the test.

Bromsulphalein Test

Originally, this test utilized phenoltetrachlorphthalein. It was later modified by Rosenthal and White,¹ who used bromsulphalein instead of phenoltetrachlorphthalein.

Bromsulphalein is phenoltetrabromphthalein sodium sulphonate, which is excreted in the bile of normal rabbits to the extent of 85 per cent in one hour after its intravenous injection. Normally it is rapidly removed from the blood stream through the activity of the liver cells, since, when the liver is extirpated, it is retained in the blood serum almost *in toto* during the early period following its injection. These are striking advantages over phenoltetrachlorphthalein, which, when similarly injected in rabbits, is excreted in the bile to the extent of only 5 to 10 per cent in one hour, and which never reaches high concentrations in the blood.

Reagent.—

This dye is used in 5% solution and may be obtained prepared in sealed ampules from Hynson, Westcott, and Dunning, Baltimore, Maryland.

Technic.—

Patient is weighed and the dosage calculated on a basis of 2 mg. per kilogram of body weight. The body weight of the patient in pounds divided by 55 will give the exact quantity, in cubic centimeters, of the 5 per cent solution required. It may be measured by drawing into a sterile 5 c.c. syringe and then slowly injected directly into an arm vein. The injection should be sufficiently slow to occupy one minute, and care should be taken not to allow infiltration of the dye outside the vein. Thirty minutes after injection, a sample of blood, 4 or 5 c.c., is drawn, preferably from the opposite arm, by allowing the blood to run through a needle directly into a dry test tube. In cases of early liver disease it may be advisable to obtain, also, a sample of blood at exactly five minutes after injection.

After coagulation, the blood is centrifuged and the clear serum is pipetted into two small test tubes. To one of these is added 1 or 2 drops of a 10 per cent solution of sodium hydroxide to bring out the color of the dye, and to the other tube a drop of 5 per cent hydrochloric acid to clear the serum of any hemolysis. The amount of dye present is now estimated by direct comparison with a series of standards.* The tube of clear acidified serum is placed in front of the standard in a suitable comparator box, and by simultaneously looking through both tubes a comparison can be made with the colored alkalized serum. The standards may be prepared by adding 4 mg. of bromsulphalein to 100 c.c. of water alkalized with 0.25 c.c. of a 10 per cent solution of sodium hydroxide. This represents the 100 per cent standard. By proper dilutions with similarly alkalized water, ten standards are prepared, ranging from 10 to 100 per cent. Five cubic centimeters of each standard may be sealed in a small test tube, and no deterioration in color will occur for several months if they are kept in the dark.

*Permanent standards with comparator box may be obtained from Hynson, Westcott and Dunning, Baltimore.

¹Rosenthal, S. M., and White, E. C.: J. A. M. A. 84: 1112, 1925.

TABLE 21.—BROMSULPHALEIN TEST IN CASES OF LIVER DISEASE

CASE	DIAGNOSIS	PERCENTAGE OF DYE IN SERUM		COMMENT
		5 MIN.	30 MIN.	
1	Carcinoma of liver, metastatic	100	99	Progressive jaundice and vomiting for two months; tumor, right breast; liver, enlarged 3 cm., at operation showed miliary infiltration throughout; patient semicomatose; death in eight days
2	Obstructive jaundice, carcinoma of pancreas, gallstones	100	97	Deep jaundice, two months; liver 13 cm. below costal margin; section removed at operation showed fibrosis; extremely sick
3	Obstructive jaundice, gallstones, cholangitis	100	95	Two weeks' jaundice, fever; at operation, purulent fluid in gall bladder; death four days later
4	Obstructive jaundice, cholelithiasis, cholangitis	---	90	Ten days' jaundice; fever; mucopurulent fluid in gall bladder; and liver swollen with rounded edge (at operation).
5	Catarrhal jaundice; hepatitis	100	75	Onset six weeks before; liver and spleen enlarged; jaundice now beginning to clear
	(Three weeks later)	---	35	Skin clear; liver and spleen remain hardened (fibrosis)
6	Catarrhal jaundice	---	50	Jaundice for one month, now clearing, liver palpable
7	Arsphenamine jaundice	---	47	Jaundice for one month; now clearing, liver not felt
8	Biliary cirrhosis	---	45	Progressive hepatic enlargement and jaundice for three years; liver edge below umbilicus; death in two weeks; necropsy
9	Obstructive jaundice, cholelithiasis, cholelithiasis	95	35	Intermittent jaundice for three years; liver 3 cm. below costal margin; operation; stone in common duct; infection not marked
10	Catarrhal jaundice, mild	---	35	Girl, aged 18; digestive symptoms; sclerae subicteroid
11	Portal cirrhosis, early	65	--	No jaundice or ascites; liver 2 cm. below costal margin; firm
12	Acute cholecystitis, obstructive jaundice	65	30	Biliary colic and jaundice for two weeks; at operation, gall bladder enlarged and wall thickened
13	Preeclamptic toxemia	55	30	Fifth month pregnancy; headaches, vomiting, and slight jaundice for three weeks
14	Portal cirrhosis	---	22	Liver enlarged 2 cm. firm and smooth; spleen enlarged 4 cm.; ascites for two months; hyperbilirubinemia

(Table continued on following page)

TABLE 21.—CONT'D

CASE	DIAGNOSIS	PERCENTAGE OF DYE IN SERUM		COMMENT
		5 MIN.	30 MIN.	
15	Carcinoma of liver (metastatic)	55	20	Aged 53; loss of weight; digestive symptoms for one year; liver to umbilicus, nodular; no ascites, no hyperbilirubinemia
16	Portal cirrhosis	---	22	Ascites for eighteen months; enlarged spleen, subicteric skin
17	Acute cholecystitis, cholelithiasis	90	10	Acute attack of vomiting and abdominal pain; skin subicteroid; at operation, mucopurulent fluid in gall bladder
18	Chronic cholecystitis, gallstones	65	5	Repeated attacks of vomiting and epigastric pain during past three years; sclerae subicteroid; gall bladder palpable
19	Syphilitic cirrhosis	50	3	Ascites for four months; liver enlarged 2 cm., irregular and nodular; Wassermann strongly positive; no hyperbilirubinemia; ascites disappeared entirely after six tapings
20	Congenital syphilis, ars- phenamine jaundice	---	22 (15 min.)	Jaundice of three weeks' duration, now fading; onset five weeks following twelfth arsphenamine injection; liver enlarged 2 cm.

Normally following the intravenous injection of 2 mg. of the dye per kilogram of body weight, it is completely removed from the blood in thirty minutes. In liver disease it is retained in the blood in concentrations that vary from 0 to 100 per cent of the amount injected. The percentage of dye present in the serum thirty minutes after injection expresses directly the degree of impaired function shown by this test.

Citing tests made by Rosenthal and White, the data in Table 21 enable one to estimate the situation as to liver dysfunction.

Improved Technic for Bromsulphalein Test

In 1938 and 1939^{1, 2} Macdonald reported a group of cases studied by the *serial* bromsulphalein method. This work confirms the validity of the *serial* method. In 1942, Mateer, Baltz, Marion, and Hollands³ modified still further the bromsulphalein method of Macdonald.

Inject 2 mg. of bromsulphalein dye per kilo body weight, the same dose as employed by Rosenthal and Macdonald. Take a blood specimen from the opposite arm every 5 minutes for a 30-minute period. A single venipuncture is done for the withdrawal of the six blood specimens of 4 c.c. each. The needle, with a three-way stopcock attached, is connected with a gravity apparatus containing normal saline solution. Between successive withdrawals of blood, the salt solution is allowed to run slowly into the vein to prevent clotting. (Two of these serial tests may be conducted at the same time by a specially trained nurse, sitting between the two reclining patients in the technic room.

¹Macdonald, Dean: Canad. M. A. J. 39: 556-560, 1938.

²Macdonald, Dean: Surg., Gynec. & Obst. 69: 70-82, 1939.

³Mateer, J. G., Baltz, J. I., Marion, D. F., and Hollands, R. A.: Am. J. Digest. Dis. 9: 13-29, 1942.

In collecting the blood specimens there is ample time for the nurse to alternate from one patient to the other, each blood specimen being collected at exactly the right time.) The per cent of dye remaining in each blood serum specimen is determined by the colorimetric method used in the Rosenthal test.

The finally improved bromsulphalein technic of Mateer et al.¹ entails a 45-minute normal standard time, using 5 mg. of the dye per kilogram dose. These writers maintain that the 30-minute period is not sufficient for proper reading of this test because in some normal individuals the dye does not disappear in 30 minutes. In other words, while the *serial* method was preferred by them, the 45-minute interval, advocated in their latest publication, for normal disappearance of the dye from the blood with the 5 mg. per kilogram dose constitutes a new standard "which, as will be noted, provides a test as sensitive as either the cephalin test or the intravenous hippuric acid test and at the same time a test which is just as reliable." Their 45-minute technic is satisfactory since this correct normal standard has been determined by the serial method. A single specimen affords the necessary information, provided it is taken exactly at the proper time. A compromise between the serial and single specimen method, which would constitute the most desirable method from a comprehensive point of view, would consist in obtaining 45-minute and 60-minute blood specimens with separate venipunctures. This would avoid the need for giving intravenous saline solution to keep the needle open, when using the serial method and obtaining a number of blood specimens. At the same time, obtaining a 60-minute specimen in addition to the 45-minute specimen would supply supplementary data regarding the degree of impaired liver function in those cases presenting evidence of impairment in the 45-minute specimen. Thus the further rate of excretion of the dye can be demonstrated in this group of cases.

The only objection to the 5 mg. per kilogram dose of bromsulphalein is that some patients have a transient reaction to this larger dose of dye. However, they believe that such a reaction develops in certain cases regardless of whether the injection of the dye is or is not followed by the injection of isotonic solution of sodium chloride and the employment of the serial method. The reaction may consist of transient headache, a feeling of faintness, and, in some cases, a chill. These symptoms usually occur from forty-five to sixty minutes after the injection of the dye. Headache may persist for several hours. No unfavorable or prolonged aftereffects were seen by them. They concluded that in the determination of the presence or absence of a slight degree of impaired liver function, two or three of the most sensitive tests should be employed; e.g., cephalin test, the 5 mg. per kilogram 45-minute bromsulphalein test, and the intravenous hippuric acid test.

Bromsulphalein, Spectrophotometric Method²

Principle.—

The bromsulphalein is injected intravenously, and later the quantity in the serum is measured colorimetrically. The dye is removed from the blood stream by the liver; therefore, this is a test of liver function.

¹Mateer, J. G., Baltz, J. I., Marion, D. F., and MacMillan, J. M.: *J. A. M. A.* **121**: 723-728, 1943.

²Consolazio, C. F., Johnson, R. E., and Marek, E.: *Metabolic Methods*, St. Louis, 1951. The C. V. Mosby Company.

Reagents.—**Bromsulphalein Dye.—**

This is purchased. A 3 c.c. ampule contains 150 mg.

5% Hydrochloric Acid Solution.—

Add 50 c.c. concentrated hydrochloric acid, c.p., to distilled water in a liter volumetric flask and dilute to 1,000 c.c. with distilled water.

N/10 Sodium Hydroxide Solution.—

See page 25.

Equipment.—

Coleman Junior Spectrophotometer, Model 6.

19 by 150 mm. cuvettes.

15 c.c. round-bottomed centrifuge tubes.

5 c.c. syringes with 20-gauge needles, sterile.

1.0 c.c. syringe pipette.

Technic.—

Weigh the patient and calculate the quantity of dye to be injected, on the basis of 5 mg. per kilogram of body weight.

Measure the dye in a 5 c.c. syringe.

Withdraw a control specimen of blood from a vein.

Separate the syringe from the needle and connect the syringe containing the dye. Inject the dye slowly into the vein, taking approximately 1 minute for the injection. *Take care not to allow infiltration of the dye outside the vein, as it is very painful!*

Record the time of injection.

After 45 minutes, withdraw a specimen of blood, 4 to 5 c.c., preferably from a vein in the other arm.

Place the blood in a clean dry test tube, and allow to clot.

Centrifuge and separate the serum from the clot.

Into each of 2 cuvettes, pipette exactly 0.5 c.c. serum, one containing serum from the blood collected before the injection, the other from serum collected 45 minutes after the injection. The specimen taken before the injection is the control.

To the control tube, add 2.5 c.c. distilled water

and 3 c.c. of 5% hydrochloric acid. Mix.

To the test serum, add 2.5 c.c. of distilled water

and 3 c.c. of N/10 sodium hydroxide solution.

Set the control at 100% transmission at 565 m μ , and read the unknown.

Calibration Curve.—

Prepare a dilution of the dye in dilute sodium hydroxide in the range of 0 to 0.1 mg. per 6 c.c.

Using a water blank set at 100% T, read the standards at a wave length of 565 m μ .

Calculation.—

A value of 10 mg. of bromsulphalein per 100 c.c. of serum is arbitrarily taken as 100% retention, when the dosage is 5 mg. of bromsulphalein per kilogram of body weight.

$$\text{Per cent retention} = \frac{\text{mg. in cuvette} \times 100 \times 100}{\text{c.c. serum in cuvette} \times 10}$$

Example.—

The per cent T was 91, corresponding to 0.02 mg. bromsulphalein in the cuvette.

$$\text{Per cent retention} = \frac{0.02 \times 100 \times 100}{0.5 \times 10} = 40\%.$$

Near-Fatal Reactions to Bromsulphalein

Certain mild allergic reactions to bromsulphalein have been reported as noted before. A report by Leon V. McVay, Jr.,¹ of a near-fatal reaction to bromsulphalein, calls attention to the exercise of greater care in performing this test.

¹J. A. M. A., Aug. 22, 1953.

This was the case of a 60-year-old Negro man in whom, after a second injection of the drug for this test (first test on Jan. 14, 1952, and the second test on April 28, 1952), certain serious symptoms appeared. Five milligrams of sulphobromophthalein per kilogram of body weight had been injected intravenously. Three minutes were consumed in making the injection. Immediately the patient complained of dyspnea and became quite apprehensive, with choking sensations, cold perspiration, pruritus, and several vomiting spells. There was an increasing pulse rate, 94 to 136 per minute, and a falling blood pressure, followed by coma. The patient was given oxygen and several injections of epinephrine hydrochloride in 1:1,000 solution. He was hospitalized and released after four days with no evidence of any sequelae.

This patient had been given these tests as a subject, with a number of others, on geriatric study. On Jan. 15, 1953, an intradermal test was made by injecting sulphobromophthalein diluted with equal parts of isotonic sodium chloride solution, and 0.1 c.c. of this mixture containing 2.5 mg. of the dye into the volar aspect of the right forearm. Within 10 minutes, an irregular area of erythema was present on the right forearm, which increased in size. The patient had a slight choking sensation. During the next 2 days, the reaction around the intradermal injection of dye increased progressively and reached maximum intensity at 48 hours, with swelling of the entire forearm. There was a slight fever during this period. Gradual remissions occurred.

This experience should convince anyone who is applying this drug *more than once* to be sure to make a precautionary intradermal test before the second injection in order to avoid any sensi-allergic manifestations. Besides, in all cases, the dye should be administered cautiously over a period of at least five minutes.

Hippuric Acid Test¹

Quick discovered that excretion of hippuric acid goes on at a constant rate; further, by increasing the amount of glycine in the diet, one may increase the output of hippuric acid. Therefore, hippuric acid elimination is largely dependent upon the speed with which the human body synthesizes glycine. Knowing that the liver is the principal organ concerned with the synthesis of glycine, Quick assumed that with liver damage there would be a corresponding diminution in the excretion of hippuric acid and that the hippuric acid test, which was once employed as a test of renal function, actually is a test of hepatic function. In this test, the physiology is about as follows: The synthesis of hippuric acid is a process of detoxification which is brought about by the conjugation of benzoic acid and glycine. This end product is eliminated in the urine as hippuric acid, a small portion only being conjugated with glucuronic acid and eliminated as glucuronic acid monobenzoate. The rate of synthesis of hippuric acid is determined by the ability of the organism to produce glycine. The output of hippuric acid is determined by the amount of liver damage. In other words, the amount of hippuric acid eliminated after sodium benzoate is administered to the patient will be diminished when there is lack of glycine to combine with benzoic acid.

¹For another test see Vol. II, Chapter XV, Toxicologic Technic, under "Toluene."

Technic.*—

The Boyce technic is the modification of the Quick method using a single 4-hour determination, which is as accurate as and far simpler than the original Quick method. It reduces the work in the laboratory and removes the objection that catheterization may be necessary to secure the hourly specimens at the appointed time. The specimen may be stored in the refrigerator for hours, or even for several days, without affecting the accuracy of the test.

Oral Test.—At 7 A.M. (the test should always be done as early in the morning as possible) give the patient a cup of coffee, with or without milk as preferred, and a slice of toast. Do not give water.

At 8 A.M. (1 hour later) give 5.9 gm. of sodium benzoate dissolved in 30 c.c. of water, to which a teaspoonful of cherry syrup has been added to make the dose more palatable. In the same glass (to be certain that all the sodium benzoate solution is taken) give half a glass of water. Give no food or fluid of any kind thereafter during the test.

Have the patient void immediately after the dose has been taken; discard this specimen. Save as a single specimen all the urine voided from 8 A.M. to 12 noon (4 hours), including the specimen voided at the conclusion of this period. If the patient has been unable to void, catheterization should be done at the end of the test.

Intravenous Test.—At 7 A.M. give the breakfast of toast and coffee as described above. If the patient is vomiting, or cannot take solid food, the entire meal, or the toast, may be omitted as necessary.

At 8 A.M. (1 hour later) inject intravenously 20 c.c. of a sterile aqueous solution containing 1.77 gm. of sodium benzoate.

Have the patient void immediately and discard the specimen. Save as a single specimen all the urine voided during the next hour (8 to 9 A.M.) including the urine voided at the conclusion of the period. Catheterization should be done at this time if the patient has been unable to void.

The intravenous test should be substituted for the oral test for patients who are nauseated or vomiting, who are too ill to be kept off fluids for the 5-hour period necessary in the oral test, or for any other reason. The beakers and syringes containing the sodium benzoate solution should be returned to the laboratory unwashed, to be certain that the whole solution has been swallowed or injected.

Measure the total amount of urine. Transfer the whole specimen to an Erlenmeyer flask. Stirring vigorously all the time, add concentrated hydrochloric acid until the urine changes Congo red test paper to blue. Continue to stir vigorously for 3 or 4 minutes more, then allow the specimen to stand at room temperature for an hour. At the end of this time, filter the precipitate off on a small Buchner funnel, using filter paper *which has previously been weighed*. Allow the precipitate to dry thoroughly in the air. Weigh.

If necessary, the drying process may be hastened in the incubator. If it is not convenient to perform the test immediately, the urine may be kept in the refrigerator for several hours, or for a day or two, without affecting the results. Specimens larger than 125 c.c. for the intravenous test and 500 c.c. for the oral test should be concentrated by boiling, having first been made acid with acetic acid.

In Quick's original test it is assumed that 100 c.c. of urine will dissolve 0.33 gm. of hippuric acid; 0.68 is the factor for changing hippuric acid to benzoic acid.

Calculation.—

$$\left(\frac{\text{Amount of urine in c.c.}}{100} \times 0.33 + \text{weight of precipitate}^\dagger \right) \times 0.68 = \text{amount of sodium benzoate as benzoic acid.}$$

Normal Values:

Oral test, 3.0 gm. of sodium benzoate as benzoic acid.

Intravenous test, 0.7 gm. of sodium benzoate as benzoic acid.

*Boyce: Role of the Liver in Surgery, Courtesy Charles C Thomas, Publisher.

†Total weight - weight of filter paper.

Tests Dependent Upon Metabolic Changes

There are a number of liver functional tests which are based upon metabolic changes, notably the galactose tolerance test and the levulose tolerance test. It must be remembered that the liver converts sugars into glycogen which is stored in the liver. This glycogen is called out from the liver by the needs of the body, being reconverted into sugar. Thus the liver is the organ that is probably the storehouse for sugar in the body.

In order to use a sugar for the determination of hepatic function, it must be one that can be recovered from the urine or blood of the patient, also one that forms glycogen when it is taken into the liver but yet offers difficulty in its conversion back into sugar. In other words, it must make the liver do considerably more work than is done in the case of dextrose. It must also possess the quality of not being utilized by muscle and therefore must be one that is not called forth by muscular effort.

The estimation of the amount of galactose in the blood rather than in urine, after ingestion of a definite amount, would seem to be the preferable procedure if a renal threshold for galactose could be demonstrated. There is convincing evidence, however, against the existence of such a threshold. Folin and Berglund¹ first furnished adequate evidence of its absence. They found that very small doses of galactose produced an increase of urinary reducing substances. Later Goldblatt,² studying the blood sugar after galactose administration, observed urinary excretion of galactose in the absence of any rise in blood sugar. Harding and Grant³ from studies on arterial blood galactose, were convinced that galactosuria can occur with very little rise in blood galactose.

In the absence of a kidney threshold, the blood level of this sugar, so far as the galactose tolerance test is concerned, is of academic interest only. As stated by Shay and Fieman,⁴ none of the advocates of blood galactose estimations has thus far demonstrated that these are more significant than the measurement of the urinary excretion of this substance in the application of this test to the differential diagnosis of jaundice. "We can, therefore," they state, "see no reason for converting a valuable laboratory test from a simple office procedure into one almost prohibitive for routine clinical use." In other words, Shay and Fieman contend that a test is best made by estimating the urinary excretion, after a standard dose of galactose, rather than by estimating the blood galactose content.

A fixed amount of galactose should be used. The blood sugar curve after galactose may be estimated, but we recommend a determination of the urinary galactose rather than the blood galactose. Shay and Fieman⁴ believe that in the clinical application of the test the time at which the test is performed in relation to the time of appearance of the jaundice is of utmost importance. The test has its greatest value and the results are most reliable when it is performed

¹Folin, O., and Berglund, H.: *J. Biol. Chem.* **51**: 213, 1922.

²Goldblatt, M. W.: *Biochem. J.* **19**: 948, 1925.

³Harding, V. J., and Grant, G. A.: *J. Biol. Chem.* **99**: 629, 1933.

⁴Shay, Harry, and Fieman, Philip: *Ann. Int. Med.* **10**: 1297-1303, 1937.

soon after the appearance of the jaundice. The longer the duration of the jaundice before the test is done the more frequently will the results be misleading.

Furthermore, the test has its greatest value in the painless jaundice group of middle and later life. If used early and judiciously in this group it will be of immense help in prognosis and will help prevent unnecessary surgery in the hepato-cellular jaundice of those age periods.

In the following methods, therefore, for the galactose tolerance test, while giving the technic of blood sugar determination, we recommend only the urinary test for determination of galactose tolerance. Emphasis must be laid upon the fact, too, that there is a possibility of glucose excretion after the dose of galactose is taken in some disturbed endocrine states and, therefore, the differential fermentation of the urine with properly prepared yeast should be made part of the routine technic in all cases in which the excretion of reducing substances in the five-hour urine exceeds 3 grams.

Galactose Tolerance Test

This test was first described by Bauer.¹

Technic.—

Dissolve 40 gm. of galactose in 500 c.c. of water and lemon juice. Administer to the patient in the morning after the bladder has been emptied.

Patient must drink water freely.

Collect hourly specimens of urine for five hours.

Combine those specimens which are positive, and calculate the total amount of sugar by the Benedict quantitative test. Proof that the sugar is galactose is made by yeast fermentation test.

Make a 10% suspension of washed brewer's yeast in one part of the urine sample.

Add 7 parts of the 10% yeast suspension. After forty-five minutes' incubation, the dextrose will have been destroyed by fermentation. Now filter and determine the amount of sugar present in the filtrate. This is galactose.

It is vital in this test to know that the Benedict reagent has been properly made. Check this Benedict reagent with a glucose solution. If, for instance, one finds that 25 c.c. of the Benedict reagent are reduced by 50 mg. of glucose, and 25 c.c. are reduced by 65 mg. of galactose, the factor for conversion would be

$$\text{glucose : galactose} :: 50 \text{ mg.} : 65 \text{ mg., or } 1:1.3.$$

The factor for conversion would, therefore, be 1.3.

Regarding the urinary findings with the galactose tolerance test: normally, less than 3 gm. of sugar are excreted in the five-hour test period. Anything over 6 gm. is considered positive and very high values indicate serious liver disturbance. Values over 3 gm., but less than 6 gm., are borderline cases.

Intravenous Galactose Tolerance Test.—This procedure is useful in differentiating parenchymatous or "medical" type from the obstructive or "surgical" type of jaundice. In a normal group, all galactose should be removed from the blood in 75 minutes. In most jaundiced patients some galactose is present in the blood at the end of the test, but the amount is strikingly different in the two types of jaundice, "medical" jaundice and "surgical" jaundice. Patients with acute hepatitis average 45 mg. per cent of galactose while those with extrahepatic biliary obstruction of less than six months' duration average

¹Bauer, R.: Med. Klin., No. 41, 1926.

only 14 mg. per cent. If 20 mg. per cent of galactose is chosen as the dividing line, 90 per cent of the cases of hepatitis are above, and 83 per cent of the cases of obstructive jaundice are below this line. In patients with obstructive jaundice of over six months' duration, the secondary liver damage robs the test of its value in differential diagnosis. Moreover, if obstructive jaundice develops in patients with pre-existing parenchymatous disease of the liver, the test shows a confusingly high blood level of galactose.

Levulose Tolerance Test

This sugar tolerance test was originally described by Strauss, later modified by Tallermann. Give 45 gm. of levulose by mouth in 200 c.c. of water, taking a blood sample before administration of the levulose for control purposes. Take blood samples at 30, 60, 90, and 120 minutes. Determine the quantity of sugar in each specimen.

Normally, there is a rise in blood sugar of 8 to 15 mg., the level never going above 130 mg. per 100 c.c. of blood and this returns to normal within two hours. In general, in severe hepatitis cases the value goes up considerably over 130, sometimes as much as 200. A rise is noted particularly in cirrhosis of the liver.

There are other tests of protein metabolism which may be performed for the estimation of liver function. An estimation of blood urea nitrogen and amino acid nitrogen may be made, of course, in serious liver disturbances. The normal ratio of urea nitrogen to amino acid nitrogen is 2 to 1. A decrease in this ratio, that is, a fall in blood urea nitrogen with increase in amino acid nitrogen, indicates severe hepatic disturbance. A falling urinary urea has been noted also in liver disease.

Takata-Ara Reaction

This test has been originated especially for the purpose of differentiating cirrhosis of the liver from other conditions which resemble it. It depends upon an increase in plasma globulin.

Technic.—

The test is made by putting 1 c.c. of 0.9% physiologic salt solution into each of a series of eight small test tubes. The first tube receives 1 c.c. of patient's serum or ascitic fluid. A series of dilutions is made by mixing and transferring 1 c.c. of the mixture to the next tube, such as is done in Lange's colloidal gold test. Add to each tube 0.25 c.c. of 10% sodium carbonate solution and 0.3 c.c. of Takata's reagent. Mix and observe immediately, after a half hour, and after 24 hours.

Takata's reagent is made by adding to one part of 0.5% mercuric chloride solution one part of 0.025% solution of aqueous fuchsin.

A positive reaction is indicated by definite precipitation in 2 of the first 3 tubes. Precipitation in the last tubes is not significant.

The value of the Takata-Ara reaction has been thoroughly studied by a number of authorities, including Israel and Reinhold.¹ They believe that the Takata-Ara reaction may be advantageously used as a preliminary or a confirmatory test of liver insufficiency but, in the detection of early cirrhosis, it is somewhat less sensitive than the dye test. Positive in most cases of advanced cirrhosis, it is often positive as well in other conditions extensively involving the liver, and cannot be relied upon to differentiate these

¹Israel, H. L., and Reinhold, J. G.: *J. Lab. & Clin. Med.* 23: 588-595, 1938.

conditions from cirrhosis. Crane's² conclusion that the chief merit of the test lay in its ability to distinguish cirrhosis from tumors was not substantiated by Israel and Reinhold, for positive reactions were frequent in neoplastic disease of the liver. Although lacking specificity for hepatic disease, positive reactions were misleading only in a tuberculous patient who was thought to have an incidental cirrhosis; necropsy revealed no lesion of the liver. Two cases of subacute nephritis with anasarca considered to be possible cirrhosis gave positive Takata-Ara reactions, but the correct diagnosis was established without difficulty. A common problem is that of differentiating the ascites of cirrhosis from that of congestive heart failure. Here, where bromsulphalein retention is, as a rule, marked and may be interpreted erroneously as indicating cirrhosis, the Takata-Ara test is less often misleading; a positive reaction is suggestive of cirrhosis.

In a comparison of various protein tests of liver function, such as total serum cholesterol, bromsulphalein, and icterus index, serum albumin was lowered and globulin increased in liver diseases in general. Although the Takata-Ara test seems a more dependable guide to the recognition of liver lesions, nevertheless diminution or inversion of the albumin-globulin ratio should be recognized as a common manifestation of diffuse liver disease. It must be remembered, too, that the icterus index is infrequently elevated in the absence of liver injury. Chronic liver disease may often show bromsulphalein retention, a positive Takata-Ara reaction, and inversion of the albumin-globulin ratio with the icterus index normal. Concentration of total cholesterol in serum is not altered in cirrhosis, although low values are common.

The Cephalin-Cholesterol Test for Liver Function, Method of Hanger

Hanger has described an interesting serologic test to differentiate obstructive from hepatogenous jaundice by flocculation of cephalin-cholesterol emulsions.

This test is made by dissolving 100 mg. of sheep brain cephalin* and 300 mg. of cholesterol in 8 c.c. of ether. An emulsion is prepared by adding 1 c.c. of the stock ether solution to 35 c.c. of freshly distilled water warmed to 65° to 70° C. Boil. This disperses all clumps. Add 1 c.c. of the emulsion to a test tube containing 0.2 c.c. of the patient's serum diluted with 4 c.c. of normal saline. Shake. Allow to stand. Note flocculation at the end of 24 and 48 hours. Grade reactions in terms of 0, plus-minus, and 1 plus, 2 plus, 3 plus, and 4 plus. Four plus indicates complete flocculation. A control tube containing 4 c.c. of saline solution and 1 c.c. of emulsion, without serum, should be made to test the stability of the emulsion. New emulsion should be prepared freshly on the day of the test. Clean glassware is to be used.

Note: Serum refrigerated one day or longer may yield false positive tests.

Precautions to Observe in Performing the Test.—Excessive bacterial contamination of the test antigen emulsion or of the serum-saline-antigen test mixture may give rise to falsely positive flocculation and accordingly may be overcome by adding sodium ethyl-mercuri-thiosalicylate in a concentration of 1:10,000 to the test antigen emulsion and to the saline used for diluting the serum. The use of clean, dry, sterile glassware in which to perform the test is rec-

*Bacto Cephalin Cholesterol Antigen for the Hanger flocculation test, as prepared by the Difco Laboratories, Detroit, Michigan, has been found excellent for this test. It is packaged in convenient containers, each unit of which is sufficient for the preparation of 5 c.c. of stock ether antigen and 150 c.c. of the final test antigen.

²Crane, M. P.: *Am. J. M. Sc.* 187: 705, 1934.

ommended. A publication by Neefe and Reinhold,¹ confirmed by Hanger and Mateer, calls attention to photosensitivity as a cause of falsely positive flocculation reactions. It was suggested that the test antigen emulsion and the serum be protected from light and that the serum-saline-antigen test mixture be incubated in the dark at room temperature (20° to 25° C.). The patient's serum should not be diluted until the test is to be performed.

Frisch-Quilligan Method for the Modified Cephalin Cholesterol Test of Hanger

In addition to these precautionary measures, Frisch and Quilligan² reported a modification of this test. They prepared a modified antigen.

Frisch and Quilligan Antigen.—

Add 1 c.c. of a stock etherized cephalin cholesterol mixture (Difco) dropwise and with agitation to 35 c.c. of redistilled water at room temperature (20° to 25° C.). Then allow the emulsion to boil slowly until the total volume has been reduced to 30 c.c., gradually cool to room temperature, and use this in the test.

This new preparation is more opalescent and the particles are more coarsely dispersed. Furthermore, serum sterol mixtures produce a granular precipitate rather than the flocculent type usually obtained with the Hanger antigen.

Technic.—

Add 0.2 c.c. of serum to 4 c.c. of 0.85% saline.

Shortly thereafter, add 1 c.c. of cephalin cholesterol antigen, thoroughly mix the reagents, and place in the dark room at 20° to 25° C.

Examine in subdued light at the end of 24 and 48 hours. Record readings from 0 to 4 plus after the method of Hanger.³ (See page 347.)

Titration.—

Make serial dilutions of serum in 0.85% saline in chemically clean serologic tubes as follows: 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256. Use a single pipette for the entire series of dilutions. To obtain a final volume of 0.8 c.c. in each tube, place 1.2 c.c. of saline in the first tube and 0.8 c.c. in each of the remaining 6 tubes. Add 0.4 c.c. of serum to be tested to the first tube, mix thoroughly, and transfer 0.8 c.c. to the second tube, mix thoroughly, transfer 0.8 c.c. to the third tube, and continue in this manner to the last tube. Mix and discard 0.8 c.c. from the last tube.

Add 0.2 c.c. of the modified antigen to all tubes.

Shake the mixture thoroughly and then place in the refrigerator at 5° C.

Examine after 18 to 20 hours. Indicate the intensity of the reaction as follows: 1 plus, a readily visible granularity of the emulsion; 2 plus, an increased granularity with or without slight sedimentation; 3 plus, heavy granulation of the particles accompanied by 50 to 75 per cent sedimentation; 4 plus, complete agglutination and sedimentation of the emulsion. Occasionally, with normal sera, the tubes containing the 1:4 and 1:8 dilutions show simple sedimentation without the characteristic conglomeration of particles when the settled material is stirred up. Such sediments are classed as negative reactions.

Interpretation of Titration Test.—Record the degree of flocculation in each tube of the serial dilution after incubation at 5° C. for a period of 18 to 20 hours. Then add the total number of plusses. This constitutes a cumulative score. The maximum score for a 7-tube titration is 28, provided all tubes show 4 plus flocculation. Conversely, the minimum score is 0. For best interpretation of results the degree of flocculation in each tube should be reported together with the cumulative score. For this purpose a graph can be readily constructed.

¹Neefe, J. R., and Reinhold, J. G.: *Science* **100**: 83, 1944.

²Frisch, A. W., and Quilligan, J. J.: *Am. J. M. Sc.* **212**: 143-153, Aug., 1946.

³Hanger, F. M.: *J. Clin. Invest.* **18**: 261, 1939.

Cephalin cholesterol emulsions prepared by the Hanger technic are flocculated by normal sera which have been diluted and exposed to light or permitted to age. The modified emulsion is not flocculated with such normal sera. It does, however, flocculate satisfactorily in the presence of sera from patients with parenchymatous liver diseases. Tests made with serial dilutions of serum and utilizing the modified emulsion permit more accurate interpretation of the flocculation pattern in liver disease than does the procedure of Hanger.

With normal human sera the emulsion remains as a stable homogeneous suspension, but with sera from patients with diffuse hepatitis, the lipoid material tends to flocculate and to precipitate to the bottom of the tube. This test is of considerable value prognostically in cases of hepatitis. Patients with mild attacks develop negative reactions before the icterus has completely subsided. A negative flocculation reaction late in the course of the disease is a favorable indication, since no relapses or evidence of persisting liver disease have yet been observed in a case of acute hepatitis once the reaction has returned to normal. The maintenance of a strongly positive flocculation reaction, irrespective of changes in the degree of jaundice, is of grave import and is usually indicative of active progressive liver degeneration.

In relation to this test, the following communication from Hanger, quoted from Mateer et al.,¹ is pertinent. "In answer to your question regarding the significance of 1+ reactions in normal individuals, I can say that it depends entirely upon the sensitiveness of your cephalin preparation. We have found that if the cephalin is put into ether soon after preparing, before it has turned dark and gummy, a weakly positive reaction is not unusual in normal individuals, particularly if the room is kept at low temperature. In the last year, however, since we have employed only a cephalin which has been ripened in air and sunlight for about six weeks, a positive reaction is practically unknown." (Hanger here obviously refers to false positive reactions.) Hanger then continues as follows: "If you use a relatively insensitive cephalin, as I have suggested, I feel that a 1+ reaction is probably of clinical significance. The interpretation of your studies should be based upon the sensitivity of the emulsion used."

Mateer et al.² believe that freshly made unripened cephalin is not sensitive enough to yield positive tests in a number of cases in which clinical or other evidence of impairment of liver cell function exists. The cephalin which has ripened in an icebox for four or five months is preferable. On the other hand, cephalin which has stood in the sunlight in an unstoppered bottle for a few weeks yielded numerous 2- and 3-plus false positive tests. Cephalin provided by the Difco Company yields uniformly satisfactory results.

Sulpho-Phospho-Vanillin Reaction of Chabrol, Charonnat, and Blanchard

Sulpho-phospho-vanillin reaction of Chabrol, Charonnat, and Blanchard is useful. It is made by using 0.1 c.c. of the fluid to be examined added to 4.9 c.c. of concentrated sulphuric acid with a specific gravity of 1.84. The mixture is placed in a boiling water

¹Mateer, J. G., Baltz, J. I., Marion, D. F., and Hollands, R. A.: *Am. J. Digest. Dis.* 9: 13-29, 1942

²Mateer, J. G., Baltz, J. I., Marion, D. F., and Hollands, R. A.: *J. A. M. A.* 121: 723-729, 1943.

bath for 10 minutes. After cooling, 0.4 c.c. of the mixture is added to 3.6 c.c. of concentrated phosphoric acid with a specific gravity of 1.71 and then 1 c.c. of a 0.6 per cent watery solution of vanillin is added. Pink color develops gradually; during the tenth minute the intensity of the color is determined colorimetrically by comparing it with the standard solutions of cobalt phosphate which the authors have suggested for the determination of cholic acid. This determines cholesterol values. **Normal values** are between 5 and 7 mg. in the blood serum, and between 20 and 30 mg. in the bile. The strongest reactions in the blood serum were found in cases of cancer of the head of the pancreas and during the development of icterogenic spirochetosis. In cirrhosis and in icterogenic hepatitis of serious character, the results were diametrically opposed. In spite of the presence of marked bilirubinemia, the sulpho-phospho-vanillin reaction was usually slightly marked. There is less color developed in toxic jaundice cases than in obstructive jaundice cases.

Blood Diastase Test

Somogyi and Gray found the normal blood diastase to be around 115. Increases of this diastase are seen in inflammatory troubles in the pancreas. In Gray's experience, high blood diastase and even higher urine diastase have been observed in acute, painful pancreatic inflammation. The figures are subnormal in cases of impaired liver function. (See pages 374 ff. for the method.)

Cholesterol and Cholesterol Ester Fraction

Esterification of cholesterol occurs in the liver. Normally the blood cholesterol level is between 150 and 230 mg. per hundred cubic centimeters. The cholesterol ester fraction is 50 to 75 per cent of the total cholesterol. The cholesterol ester fraction frequently is reduced in proportion to the extent of liver damage. Since the esterification of cholesterol is a physiologic function of the liver, it assumes a major importance as a test for liver function and may be of definite prognostic value before and after surgical interference for gall bladder disease and in the presence of certain types of liver damage.

When the cholesterol ester fraction falls below 65 per cent, damage of the liver exists.

Note that there is a discrepancy in the various values reported in the literature.

According to Portis this is an important indicator of liver function. He claims that the rise in the ester fraction parallels the clinical improvement of the patient. However, White and others¹ maintain that the ester fraction rises to a near normal level long before the hippuric acid test reveals that there is a return of normal function in the liver.

The Lange Colloidal Gold Test As a Liver Functional Test

In 1940, Gray² published his records on the value of the colloidal gold test in estimating liver function. Gray's method of standardization of gold solution entailed the use of the serum of a normal individual and that of a patient with definitely known liver disease. For subsequent tests, the amount of acid was added to the gold solution which yielded the best combination of the best negative reading with the normal serum and the best positive reading with the positive serum.

¹Tr. Am. Gastro-Enterol. A., 1939.

²Gray, Seymour J.: Arch. Int. Med. 65: 524-544, 1940.

Method of Lavin, Sellek, and Del Frade³

Obtain 5 c.c. of blood by venipuncture during fasting.

Allow to clot, loosen the clot, and centrifuge at high speed to separate serum from clot.

Make a 1:350 dilution of the serum in physiologic saline (0.1 c.c. serum + 34.9 c.c. saline).

Arrange a series of 11 tubes like those used in testing spinal fluid (page 1254).

Place 1.8 c.c. of a 0.3% sodium chloride solution in the first tube and 1 c.c. in each of the remaining tubes.

Add 0.2 c.c. of the serum dilution to the first tube. Mix well.

Take 1 c.c. from the first tube and transfer to the second tube. Repeat up to tube No. 10 and then discard 1 c.c. Tube No. 11 is used as control and contains no serum.

Add 5 c.c. colloidal gold, pH 7.0.

Let stand from 12 to 24 hours and then read.

Readings:

- 0-----No change
- 1-----Bluish-red
- 2-----Violet
- 3-----Blue
- 4-----Pale blue
- 5-----Colorless

Positive cases cause complete discoloration in the first 3 to 8 tubes or incomplete flocculation in the first 3 to 4 tubes. This gives a curve of 5543210000, 5432100000, or 4432100000. These curves are similar to those observed in the erroneously called general paretic curve. The following cases and curves from the Departments of Pathology and Pediatrics of Professor Aballi demonstrate this reaction:

1. Hepatomegaly-----	4432100000
2. Cirrhosis of the liver-----	5555543100
3. Hepatomegaly-beriberi-----	0011210000 (treated)
4. Syphilitic hepatitis-----	5555544310
5. Hepatomegaly-toxicosis gravis-----	1221100000
6. Rheumatism-cirrhosis-----	5544321000
7. Mesenteric tumor-----	5422100000
8. Nephrosis-nephritis-----	1122100000
9. Toxicosis—slight hepatomegaly-----	0001000000
10. Hepato-splenomegaly-----	1122100000
11. Acute nephritis-----	1122100000
12. Catarrhal icterus-----	1231100000

Behavior of Reaction in Normal Sera

1. -----	1132000000	11. -----	0012221000
2. -----	2444310000	12. -----	3322210000
3. -----	0221100000	13. -----	3322210000
4. -----	0001222100	14. -----	1222100000
5. -----	0121100000	15. -----	1111000000
6. -----	0121100000	16. -----	1221000000
7. -----	1232100000	17. -----	0000222000
8. -----	2221100000	18. -----	1232100000
9. -----	4443321000	19. -----	0001100000
10. -----	1222100000	20. -----	3322210000

Cases and Curves From the Department of Clinical Medicine (Ortega)

1. 5554321000—Cirrhosis.
2. 5554333220—Paresis. Serology + + + +. Takata-Ara + +.

³Lavin, Rogelio, Sellek, Antonio, and Del Frade, Alejandro: Lab. Digest 6: 8, Jan., 1943, p. 4 translated from Vida, Nueva, No. 16, No. 5, Vol. 49.

3. 1121000000—Primitive neoplasia of the stomach with multiple hepatic metastases.
4. 1212221000—Parkinson's disease.
5. 5555444320—Compression of the superior vena cava; hepatic stasis.
6. 5555554420—Sprue; fatty degeneration; no cirrhosis.
7. 0000121000—Ascites; normal biopsy.
8. 1110000000—Syphilitic (serology ++++); chronic cardiopathy; negative Takata-Ara.
9. 4332210000—Gastric scirrhus; uremia.
10. 2222100000—Chronic obstructive jaundice.
11. 0132100000—Mitral stenosis.
12. 3322210000—Chronic cholecystopathy; sub-icterus.

Takata-Ara reaction sometimes positive, others negative.

These writers believe that this test is superior in sensitivity to the Takata-Ara test. It is evident that in those cases in which the proteinogenic functions of the liver are disturbed, causing a subsequent rise of globulin in the blood, the reaction is positive. On the other hand, there are cases in which there is no increase in globulin and yet the reaction is positive, thus pointing to the fact that other factors are also involved. The reaction is not specific for any particular disease but may be positive in diverse conditions. The degree of positivity seems to be related to the functional state of the liver, and changes in intensity with improvement of the condition of the patient whenever this latter is possible. In children with functional hepatomegaly, the test was positive in 70 per cent of the cases. In two cases of catarrhal jaundice the curves were 5554321000 and 5544321000, respectively. In all four cases in adults, diagnosed by liver puncture as fatty degeneration of the liver, the reaction was positive. In a study of the reaction on a patient with yellow atrophy of the liver, as diagnosed by means of laparotomy and biopsy, the reading was 5443321000. In a patient with Wilson's disease, liver puncture showed fatty degeneration and the test gave a strongly positive reaction. The test was positive in two cases of chronic cardiopathy associated with hepatic stasis. The test was negative in all ten of a group of syphilitic sera from patients showing no evidence of hepatic lesions. In the control group of twenty cases, 5 per cent, that is, one, had to be registered as a false positive and required clinical re-examination. Summarizing, the Lange colloidal gold reaction on blood serum is one of the most sensitive tests for the study of liver function.

The Iso-Iodeikon Test

This test is one of the very important liver functional tests which have been developed by Graham and his associates at the Washington University Medical School in St. Louis. Iso-iodeikon is administered in quantities eight times that recommended for liver function tests with other dyes. This larger dose permits a more accurate determination of the liver efficiency in excreting the dye from the blood stream and a better estimate of the risk of operation.

Technic—

The dosage is at the rate of 40 mg. per kilogram of body weight and not to exceed 2.5 grams for any case.

Iso-iodeikon is dissolved in about an ounce of freshly distilled water. The solution is filtered through fine filter paper and then sterilized for 15 minutes in a boiling water bath. Solution should be freshly made, not more than 24 hours before it is used.

Injection is made by the gravity method. Place 150 c.c. saline solution or Ringer's solution in a buret and connect up with a vein through needle. Sterilize the outside of

the rubber tubing with iodine and alcohol and inject the solution of iso-iodoikon with needle and syringe through the tube after the flow of saline or Ringer's solution into the vein has been established. At this time, the rate of injection of dye can be absolutely controlled and stopped at any time if any symptoms of toxicity occur. The patency of the vein may be maintained by allowing saline or Ringer's solution to flow into the vein. The speed of the injection must be calculated so that the injection of the dye will be finished before all of the physiologic saline or Ringer's solution has run out. This precaution will insure the washing out of the vein and thereby diminish the possibility of phlebitis. The injection should not be completed in less than 15 minutes.

Use perfectly clean glassware and distilled water of known purity. Medicine glasses, syringes, etc., must be washed free from any antiseptic solutions. Be sure to have an isotonic saline solution. The distilled water must be used within 24 hours after distillation. Beware of tissue necrosis from extravasation of this drug into the extravascular tissue.

Note that iso-iodoikon and iodoikon solutions are very sensitive to even small traces of carbon dioxide. During preparation and sterilization, solutions of these compounds may become exposed to the air sufficiently long to absorb enough carbon dioxide to cause a slow precipitation of small quantities of the free iodinated phthaleins. If such a precipitation occurs, allow the solid material to settle to the bottom of the tube. The supernatant fluid is then drawn off, without agitating the solution. All rubber tubing must be boiled in a weak solution of sodium carbonate or sodium hydroxide.

Determination of Liver Function.—

Collect 8 c.c. of blood one-half hour and if desired the same quantity one hour after injection of the dye. Examine each specimen separately.

Centrifugalize to separate the serum and then remove it with a pipette, placing equal amounts in two of the test tubes supplied in the colorimeter.* Alkalinize one tube only with a small drop of a 5% sodium hydroxide solution to bring out the color of the iso-iodoikon in the blood serum. Do not add too much alkali because it produces a brownish color. Add a second small drop to see if the color can be further intensified, but do this cautiously because excessive alkali destroys the natural color and makes a comparison with the standards unreliable. The colored sample and the tube of clear serum are placed in the set for comparison with the standards furnished by the company.

Make the readings under the same conditions, holding the colorimeter up against the same light each time a determination is made. A daylight bulb or a nitrogen bulb is preferable to the usual frosted bulb, and much preferred over regular daylight which is constantly varying.

The sealed ampoule standards bear the following percentages: 5, 10, 15, 20, 25, 30, 40, 60, 80, 100. When not in use, place the lid on the box, since exposure to light causes the standards to fade.

The 100% standard represents the concentration of the dye in the blood immediately after injection before absorption. The smaller percentages give a gradation of this concentration down to 5%. By holding the colorimeter up to diffused light and matching the unknown sample with one of the standards, the amount of dye retained in the blood stream can be determined.

In this colorimeter, as in other colorimeters of this type, the operator looks through the same three media—water, dye, and serum—in each slot, thus assuring the reliability of the test.

Repeat the procedure with the second specimen taken one hour after injection of the dye, although this sample is not as important as the thirty-minute sample.

Interpretation.—A retention of about 10% of the dye one-half hour after injection and about 5% or less one hour after injection is considered normal. The greater the percentage of dye retained in the serum, the more severely impaired the liver function. The largest amounts of dye retention have been found in patients suffering from acute cholangitis and hepatitis, such as that

*Supplied by Mallinckrodt Chemical Company, St. Louis, Mo.

encountered in acute catarrhal icterus, acute hepatic necrosis, and acute hepatitis. In some cases of severe cholecystitis, the amount of retention may be as high as 80 to 90%.

The Quantitative Determination of the Thymol Turbidity Reaction of Serum

The thymol turbidity reaction of serum was described by Maclagan.^{1, 2} Ley, Lewis, and Davidson³ recommended a modification of this method using the Evelyn colorimeter. They expressed the values for turbidity as cubic centimeters of the barium sulphate suspension used as standard. The approximate Maclagan units determined visually were presented for comparison.

They found that the thymol turbidity of 105 apparently healthy young men varied from 0.16 to 2.19 c.c. of barium sulphate suspension. The upper limit of normal, using three times the standard deviation, was 1.68 c.c. In cirrhosis of the liver, 81 per cent of the determinations were abnormal; in severe cirrhosis, 92 per cent. In a series of illnesses without evidence of liver disease, 14 per cent were above normal but none above 3 per cent of barium sulphate suspension; whereas 37 per cent of the determinations in cirrhosis were above this figure.

Reagents.—

Carry out the entire reaction with serum, as well as the formation of the standard barium sulphate suspensions, in Evelyn tubes. Use a 660 γ filter in an Evelyn colorimeter. Change the galvanometer readings G to L values ($2 - \log G$). Use distilled water for the 100 setting on the colorimeter rather than the thymol buffer, as the latter tends to become turbid upon standing. Slight turbidity of the thymol buffer clears when it is added to the serum, but in general cloudy buffer should not be used.

Thymol-Barbiturate Buffer, Maclagan.²—pH 7.8, $\mu = 0.01$.

Place in a 1000 c.c. Pyrex Erlenmeyer flask

1.38 gm. of barbitone

1.03 gm. sodium barbitone

3.0 gm. (approximately) of thymol.

Add 500 c.c. distilled water

Heat just to boiling point, shake well, and cool thoroughly. The mixture should now be turbid.

Seed with a small amount of powdered thymol crystals, shake, and allow to stand overnight at a temperature of 20 to 25° C.

Again shake well, to avoid supersaturation, and filter the clear solution from the crystalline deposit. It is not possible to shorten this technic by weighing an exact amount of thymol, because there is some interaction between the thymol and the barbitone on heating, presumably esterification, and an excess of thymol must be present to ensure saturation. The solubility of thymol is significantly reduced below 20° C.; the buffer should therefore be stored in a warm place in cold weather.

Standard Barium Sulphate Curve.—

Prepare a barium sulphate suspension as described by Shank and Hoagland,⁴ using 3 c.c. of 1 per cent barium chloride diluted to 100 c.c. with 0.2 N sulphuric acid.

Make several dilutions of the standard with 0.2 N sulphuric acid, and measure the resulting turbidity in the Evelyn photoelectric colorimeter.

Make seven serial dilutions of the barium sulphate suspension, each one five times.

¹Maclagan, N. F.: *Nature*, London **154**: 670, 1944.

²Maclagan, N. F.: *Brit. J. Exper. Path.* **25**: 234, 1944.

³Ley, Lewis, and Davidson: *J. Lab. & Clin. Med.* **31**: 910, Aug., 1946.

⁴Shank, R. E., and Hoagland, C. L.: *J. Biol. Chem.* **162**: 133, 1946.

Obtain the mean value for each dilution and derive a regression equation from the data.

The equation is: $Y = 20.81X - 0.0271$, where $Y =$ c.c. of barium sulphate suspension, and $X = L$ value ($2 - \log G$).

The values obtained by Ley, Lewis, and Davidson are shown in Table 22. The curve drawn from these values is a straight line. The approximate MacLagan units corresponding to the dilutions of the barium sulphate standard are given in Table 22.

TABLE 22.—RELATION BETWEEN VOLUME OF BARIUM SULPHATE SUSPENSION, EVELYN READINGS, AND MACLAGAN UNITS

BaSO ₄ SUSPENSION (C.C.)	0.2 N H ₂ SO ₄ (C.C.)	EVELYN GALVANOMETER READING	L VALUE (2 - LOG G)	APPROX. MACLAGAN UNITS
0.5	5.5	94 ²	0.0246	2½
1.0	5.0	89 ⁰	0.0505	5
2.0	4.0	79 ³	0.0982	10½
3.0	3.0	71 ²	0.1457	16
4.0	2.0	64 ⁰	0.1936	22
5.0	1.0	57 ⁰	0.2441	28
6.0	0.0	31 ¹	0.2903	34

Technic.—

Place, in succession, in an Evelyn tube

0.1 c.c. of serum

6.0 c.c. of thymol-barbiturate buffer.

Mix well and keep at room temperature for thirty minutes.

Obtain galvanometer readings using distilled water as the 100 setting.

Change the galvanometer readings G to L values ($2 - \log G$). The corresponding cubic centimeters of barium sulphate suspension, obtained from the equation by calculation or from the curve, indicate the degree of equivalence of thymol turbidity.

Thymol Turbidity Test Set, MacLagan Procedure

A standard test set (Harleco) for this test is manufactured by the Hartman-Leddon Company of Philadelphia, and is highly recommended. It consists of a set of turbidity standards in a Plexiglas comparator. The degree of thymol turbidity is subject to close estimation with these standards, intermediate comparatives being available in the lower half of the series.

Principle.—

Varying degrees of turbidity and flocculation are occasioned in the serum of patients suffering from certain parenchymatous liver diseases upon mixture of the serum with a solution of thymol in barbitol buffer. The reaction is quantitative under standardized conditions of testing. Fig. 116.

The test is very simple. The serum is mixed with thymol buffer in the proportion of 1:60. Should the observed turbidity exceed the highest standard (10 MacLagan units), the test is repeated with a serum-buffer ratio of 1:120.

Reagents.—

Standards.—The standards consist of 15 sealed tube suspensions of predetermined values, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 MacLagan units.

The original color of these standards is retained best by exposure to daylight, and therefore the rack with the standards should be stored on an exposed laboratory shelf.

Avoid undue exposure to heat, although a maximum of 110° F. should not prove detrimental unless prolonged over long periods. Standards should be replaced after a year, but this time may be extended.

Thymol Buffer Solution.—Refer to page 354.

Technic.—

(A) Turbidity Measurement:

Measure the serum to the 1:60 calibration on the pipette (0.164 c.c.), and transfer to the special comparison tube.

Add the thymol buffer solution, pH 7.8, to the 10 c.c. mark.

Mix and allow to stand for 30 minutes.

Compare with the standards. All readings must be completed within 30 minutes.

(B) Flocculation Measurement:

Allow the tubes to stand for a further 18 hours, then grade them as 0 to 4+ according to the degree of flocculation observed:

1+ : slight but definite flocculant precipitate.

4+ : maximal flocculation, clear supernatant fluid.

2+ to 3+ : intermediate flocculation, unclear supernatant fluid.

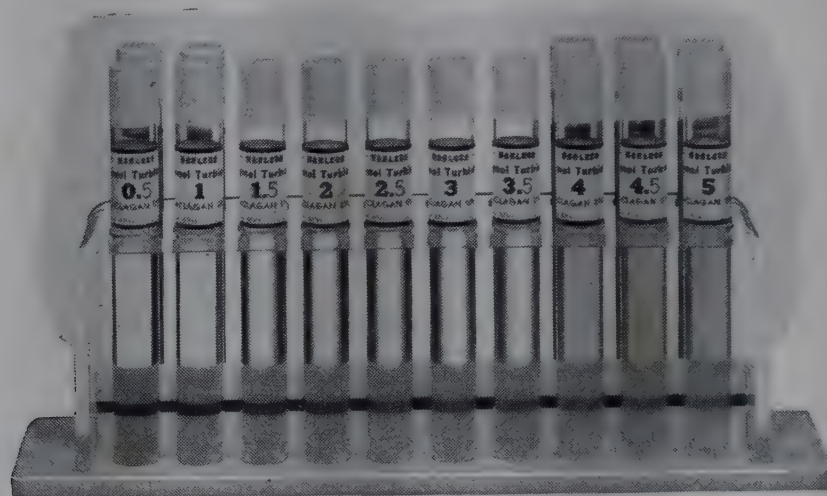


Fig. 116.—Thymol turbidity standard test for the MacLagan test. (Courtesy Hartman-Leddon Company ["Harleco"], Philadelphia, Pa.)

Calculation.—

No calculation of turbidity for expression in MacLagan units is required when comparison is made with the Harleco standards. Each standard is labeled to read directly in MacLagan units. Dilutions of the sample in ratio of 1:120 require that the labeled value be doubled.

The thymol turbidity test by means of visual comparison with these semi-permanent standards is the most reliable means of estimation. It also is the most convenient when accurate standards are available and do not need to be prepared immediately before use. The "secondary" suspension standards of graded barium sulphate have the disadvantage of particle agglomeration unless prepared frequently and freshly for the test. MacLagan units have been erroneously reported clinically from comparison with secondary standards that differed by as much as 100 per cent.

FATTY ACIDS

Modified Method of Bloor

Reagents.—

Alcohol, Redistilled.—Absolute alcohol is better. Made by adding heated anhydrous copper sulphate to 95% alcohol, and using the supernatant fluid.

Ether.—**Standard Fat Solution.—**(5 c.c. contain 2 mg. fat.)

Prepare, first, solutions of oleic and palmitic acids, each containing 200 mg. of the fatty acid in 500 c.c. alcohol.

To 60 c.c. oleic acid solution

Add 40 c.c. palmitic acid solution.

Concentrated Sodium Hydroxide.—

This should be made from sodium.

Dilute Sulphuric Acid.—

To 3 parts of distilled water

Add 1 part of sulphuric acid.

Always add the acid to the water. Add it slowly, as the solution becomes very hot.

Chloroform, Dry.—

Place in the bottom of a wide-mouth bottle of chloroform a few crystals of calcium chloride.

To use, filter through filter paper.

Dilute Hydrochloric Acid.—

To 3 parts of distilled water

Add 1 part of hydrochloric acid, c. p.

Add the acid to the water.

Equipment.—

- 1 100 c.c. volumetric flask.
- 1 100 c.c. graduated cylinder.
- 3 100 c.c. Erlenmeyer flasks, nonsoluble glass.
- 2 200 c.c. beakers.
- 1 4 inch funnel.
- Filter paper.
- 1 small funnel with long stem.
- Hardened filter paper, small size.
- Water-bath and electric hot plate.
- 4 10 c.c. volumetric pipettes.
- 3 5 c.c. volumetric pipettes.
- 2 1 c.c. pipettes graduated in 0.1 c.c.
- 1 10 c.c. graduated glass-stoppered cylinder.

Technic.—

Place in a 100 c.c. volumetric flask 75 c.c. of a mixture of 3 parts of redistilled alcohol and 1 part ether.

Add 5 c.c. blood plasma, slowly and with constant shaking of the flask.

Immerse in a beaker of boiling water over an electric hot plate.

Rotate the flask constantly to prevent overheating, and bring contents to a boil.

Cool to room temperature.

Dilute to volume with a mixture of 1 part ether to 3 parts alcohol.

Mix and filter through dry filter paper.

Place 10 to 20 c.c. (preferably 10 c.c.) of filtrate in a 100 c.c. Erlenmeyer flask, nonsoluble glass.

Add 0.1 c.c. concentrated sodium hydroxide solution (made from metallic sodium).

Evaporate nearly to dryness over a water-bath.

The process of drying is very important. Do not carry it too far, or the cholesterol cannot be extracted in the cold treatment. Carry the evaporation far enough, or a part of the soap or fatty acids is extracted with the cholesterol.

For the correct method of evaporation, the Erlenmeyer flask should be frequently rotated or gently shaken when the mixture has evaporated down to 10 drops of liquid.

Continue the drying until only a few drops remain. Shake the flask against the palm of the hand so as to distribute the fluid evenly over the bottom of the flask. If alcohol fumes are still given off, continue the evaporation; add a drop of distilled water to keep the contents moist, if necessary; but if there are no fumes of alcohol, discontinue the process.

Cool. Add 0.1 c.c. dilute sulphuric acid (1 part concentrated sulphuric acid to 3 parts distilled water).

Mix well.

If the contents are not alkaline, a more dilute acid should be used.

If the quantity in the flask is too small to insure complete mixing, add a few drops of distilled water.

Heat over a water-bath until the last traces of water have disappeared.

Extraction of Cholesterol:

Cool the flask.

Add 10 c.c. chloroform (dry).

Allow to stand 10 minutes.

Shake occasionally to reach material adherent to the sides.

Pour the chloroform extract through a small hardened filter paper into another flask from which the cholesterol estimation may be continued.

Repeat this process twice with 5 c.c. chloroform each time.

Determination of Fatty Acids:

Add 10 c.c. redistilled (or absolute) alcohol to the residue in the small Erlenmeyer flask.

Raise the contents to boiling on an electric hot plate.

Boil very gently for ten minutes.

Pour the hot alcohol mixture through the same hardened filter as was used for filtering the chloroform into a 100 c.c. Erlenmeyer flask.

Repeat this process, using 5 c.c. alcohol, and filtering into the same flask through the same filter paper.

Evaporate the combined filtrates to 2 or 3 c.c.

Transfer the evaporated filtrates quantitatively to a 10 c.c. graduated glass-stoppered cylinder, rinsing the flask with enough alcohol to bring the quantity to 5 c.c.

Place in a 200 c.c. beaker 100 c.c. distilled water.

Add the alcohol extract of the fatty acid, with stirring, through a very small funnel. The stem should be drawn out to form an opening of about 1 mm., and should extend down to the bottom of the beaker.

Rinse the cylinder once with the solution in the beaker.

Pour the rinsings back to the beaker through the small funnel.

In a 200 c.c. beaker, marked "S" for standard, place 100 c.c. distilled water.

Add 5 c.c. standard fat solution (containing 2 mg. fat).

Add 10 c.c. dilute hydrochloric acid (1 part HCl to 3 parts water) to both the unknown and the standard. **Do Not Stir.**

Allow both mixtures to stand not less than three minutes and not more than ten minutes, and read in the colorimeter.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 2.0 \times \frac{100}{0.5^*} = \text{the mg. of fat in 100 c.c. blood plasma.}$$

Example.—

Reading of standard is 15; reading of unknown is 10; 10 c.c. filtrate used.

$$\frac{15}{10} \times 0.2 \times \frac{100}{0.5} = 60 \text{ mg. fat in 100 c.c. plasma.}$$

Normal is 310 to 360 mg. per 100 c.c. blood.

*If 20 c.c. blood filtrate were used, this figure is 1 instead of 0.5.

Interpretation of Fatty Acid Changes

It is of considerable interest to know something of the amount of fat or lipoids in the blood during diabetic investigations. These accumulations in diabetes are well known and indicate pathologic changes of highest import to the clinician. This has been alluded to in our remarks upon acidosis. In addition to this condition, we might mention the interesting work of Bloor and MacPherson¹ on the blood lipoids in anemia. Since the characteristic features of anemia and pernicious anemia are destructive changes in the red cells, attention has been called to the hemolytic and the antihemolytic substances, particularly blood lipoids. The work of Gorham and Myers indicated their findings in pernicious anemia in respect to cholesterol. Berger and Tsuchiya² reported that the ether extract of the intestinal mucosa of a patient dead with pernicious anemia had several times greater hemolytic power than that of normal mucosa. McPhedran³ failed to substantiate this report. Faust and Tallqvist⁴ found hemolytic lipoids in the pancreas and gastrointestinal mucosa of persons not suffering with anemia. Kullmann⁵ and later Faust and Tallqvist⁴ found that the lipoids of cancer tissue were hemolytic. Considerable work has been done on the lipoids in anemia due to the *Diphyllobothrium latum*. In 1888 Schapiro⁶ described a form of anemia due to this worm. Tallqvist⁷ demonstrated hemolytic lipoids in this worm and later with Faust⁴ isolated the hemolytic substance which was cholesterol oleate. They found the oleic acid was the hemolytic substance. Faust found that long-continued administration of oleic acid to dogs and rabbits gave rise to anemia conditions. Since the hemolytic agent in all these experiments was the unsaturated fatty acids, it appears that toxic quantities of these acids enter the blood by way of the chyle. There are three explanations for the anemia brought about by long-continued feeding with unsaturated fats: abnormality of the absorptive mechanism, allowing certain amounts of hemolytic lipoids to reach the blood; failure of the assimilative mechanism in the blood or tissues resulting in an abnormal accumulation of these substances either free or in the form of toxic derivatives; or, a decrease in the hemolytic substances in the blood.

Cholesterol and lecithin have been shown to act antagonistically in certain types of hemolysis (by cobra venom); both are believed to take an active part in fat metabolism. However, but little attempt has been made to study these substances in relation to anemia. Bloor and MacPherson therefore undertook an investigation along these lines, mostly on patients with pernicious anemia, with a few cases of secondary anemia including one from *Diphyllobothrium latum*. They found that the blood lipid values in anemia were normal or nearly so, as long as the percentage of blood corpuscles remained above half the normal value. When the percentage dropped below this level, abnormalities appeared which, in the order of their magnitude and

¹Bloor and MacPherson: J. Biol. Chem. 31: 79, 1917.

²Berger and Tsuchiya: Deutsch. Arch. f. klin. Med. 96: 252, 1909.

³McPhedran: J. Exper. Med. 17: 527, 1913.

⁴Faust and Tallqvist: Arch. exper. Path. u. Pharm. 57: 367, 1907.

⁵Kullmann: Ztschr. f. klin. Med. 53: 293, 1904.

⁶Schapiro: Ztschr. f. klin. Med. 13: 416, 1888.

⁷Tallqvist: Ztschr. f. klin. Med. 61: 427, 1907.

also of the frequency of their occurrence, were (1) high fat in the plasma, (2) low cholesterol in the plasma and occasionally in the corpuscles, and (3) low lecithin in the plasma. The lipid composition of the corpuscles was found to be normal in almost all cases. There was therefore nothing in their composition to indicate abnormal susceptibility to hemolysis. In their studies on splenectomy cases, six in all, they found increased total fatty acids and lecithin in the corpuscles and of cholesterol in the plasma. The results were the same whether the patients had anemia or not. The relation between free and bound cholesterol was found to be within the normal limits in all cases of anemia except the two cases in which there was carcinoma, thus giving little support to the assumption that an abnormally great combination of cholesterol as ester is a factor in the production of anemia. The low values for lecithin and the high values for fat which were generally most marked in these cases where the blood corpuscle percentages were lowest are regarded as due to deficient fat assimilation in the blood resulting from the lack of sufficient corpuscles to bring about the change of fat to lecithin which has been found to be one function of the corpuscles. While the results offer no certain evidence that abnormalities in the blood lipoids are responsible for anemia, the low values for cholesterol, which is an antihemolytic substance, and the high fat fraction, which may indicate the presence of abnormal amounts of hemolytic lipoids in the blood, are possible causative factors which the writers believe may be proved by subsequent investigations.

Bloor, continuing his work on blood lipoids, has studied the facts of lipemia in connection with nephritis.¹ Previous workers have found fat disturbances in connection with this disease. Thus Watjoff² found in a case of nephritis microscopically visible fat which stained with osmic acid. Boenniger³ reported fat high. Erben⁴ showed increased values for fat and lecithin in a subchronic case. Greenwald⁵ found high lipid phosphorus in some of his nephritics. Chauffard, LaRoche, and Girgaut⁶ found hypercholesterolemia in chronic nephritis with milky plasma in a case of uremia, and Widal, Weill, and Laudat⁷ found lipemia frequently in nephritis. Henes⁸ found the cholesterol in blood increased the most in the severest cases. Mueller⁹ found high lipid values in a case of nephritic lipemia. Schmidt¹⁰ found the cholesterol values high in hypertension, when the kidney function was disturbed. Epstein and Rothschild¹¹ found the blood lipoids high in chronic nephritis. They found the lipoids diminished in uremic cases. Denis¹² found an increase of cholesterol in the blood in but one case of nephritis out of fifty examined. Bloor's figures are based upon an analysis of samples taken before breakfast so as to exclude alimentary lipemia and these samples

¹Bloor: *J. Biol. Chem.* **31**: 575, 1917.

²Watjoff: *Deutsch. med. Wchnschr.* **23**: 559, 1897.

³Boenniger: *Ztschr. f. klin. Med.* **42**: 65, 1901.

⁴Erben: *Ztschr. f. klin. Med.* **1**: 441, 1903.

⁵Greenwald: *J. Biol. Chem.* **21**: 29, 1915.

⁶Chauffard, LaRoche, Girgaut: *Compt. rend. Soc. de Biol.* **70**: 108, 1911.

⁷Widal, Weill, Laudat: *Semaine med.* **32**: 529, 1912.

⁸Henes: *Deutsch. Arch. f. klin. Med.* **111**: 122, 1913.

⁹Mueller: *Ztschr. f. physiol. Chem.* **86**: 469, 1913.

¹⁰Schmidt: *Arch. Int. Med.* **12**: 121, 1914.

¹¹Epstein and Rothschild: *J. Biol. Chem.* **29**: 4, 1917.

¹²Denis: *J. Biol. Chem.* **29**: 93, 1917.

were treated at the hospital with alcohol-ether as soon as obtained to obviate changes produced by standing. He found that the abnormalities in the blood lipoids in severe nephritis were found to be high fat in plasma and corpuscles, and high lecithin in the corpuscles. The cholesterol values were practically normal. These abnormalities he found were the same as those found in alimentary lipemia and for this reason are regarded as a result of a retarded assimilation of fat in the blood, which, in turn, is thought to be one manifestation of a general metabolic disturbance brought about by a lowered "alkali reserve" of the blood and tissues.

LIPOIDS

Serum Lipoids, Method of Mata¹

Reagents.—

Fat-Free Filter Paper.—

Roll filter paper and place in a test tube. (Use 13 cm. paper.)

Cover for several minutes with a mixture of ether and alcohol, 1 part of alcohol for each 5 parts of ether.

Mix by inverting the tube repeatedly.

Replace the ether-alcohol and repeat the process.

Dry the paper by agitating it in the air.

This paper can be used immediately upon drying, or it can be kept indefinitely.

Chloroform.—

Cholesterol Standard.—

Dissolve 20 mg. of pure cholesterol in 250 c.c. of dry chloroform.

Anhydrous Acetic Acid.—

Sulphuric Acid, c.p.—

Technic.—

Pipette 1 c.c. of oxalated or fluorated whole blood onto a fat-free filter paper, 13 cm. in diameter.

Touch the pipette to the paper and allow the contents to run from one end to the other, repeating this in the direction of the greatest diameter, with parallel blood lines so near each other that they unite when the blood flows, without leaving any white, uncovered areas.

Fold the paper, impregnated with blood, in an arch, joining the two edges which do not have blood. Hold it with a pair of forceps and place it in the hottest portion of the incubator, keeping it from contact of all kinds by means of a piece of gauze.

Keep it in the incubator until all of the humidity or "shine" of the spread-out blood has disappeared and the blood is dry. The impregnated paper may now be handled without staining the fingers.

After the drying process has been completed, cut away the margins of the paper which do not show any blood.

Fold the paper in narrow inverted folds, beginning from the narrowest end.

Place the folded paper in the bottom of an extraction tube, which is an ordinary 5-inch test tube that has been thoroughly cleaned and made fat-free with ether-alcohol mixture.

Cover the blood-stained paper with a fresh mixture of 5 parts of ether and 1 part of 95% alcohol.

Stopper the test tube with a clean, fat-free cork, and keep it stoppered for 15 minutes, shaking several times during this extraction period and rotating it in the palm of the hand.

Clean and dry a 50 c.c. Erlenmeyer flask and make it fat-free with ether-alcohol mixture and then tare it.

¹Mata, Manuel: Lab. Digest 11: 8, Jan., 1948.

Prepare a fat-free small glass funnel by plugging the stem and adding a small amount of alcohol-ether mixture, and then remove the plug.

Place this filter funnel in the tared Erlenmeyer flask.

After 15 minutes, pour the blood mixture through the filtering funnel into the Erlenmeyer flask.

Wash the tube several times with small portions of alcohol-ether and combine the washings with the extract in the tube, covering each time and agitating slowly.

Cover the paper again with ether-alcohol for 10 more minutes. Repeat the washing.

Place the Erlenmeyer flask containing any remainder of the ether-alcohol mixture in a water bath so that the water covers the lower portion of the neck, to prevent moisture in the neck of the flask. Gradually and carefully raise the temperature of the water bath until complete evaporation takes place. See below for directions.

Keep at the boiling point of the water bath for 5 minutes to insure complete dryness of the residue.

After 5 minutes, remove the flask, allow it to cool, and dry it on the outside with a moist cloth followed by a dry cloth. This is to remove all particles that might have come from the water bath and which will increase the weight of the flask.

When the flask is thoroughly clean and dry on the outside, weigh on an analytical balance and record the weight.

Calculation.—Subtract the weight of the flask from the weight obtained in the test. The difference is the number of milligrams of total lipoids in the c.c. of whole blood used. Multiply this result by 100 to obtain the total lipoids in 100 c.c. of whole blood.

Normal lipoids is 450 to 550 mg. per 100 c.c. of whole blood.

Determination of Total Cholesterol.—

After the weight of the extract has been determined, dissolve it in 5 c.c. of dry chloroform.

Place 1 c.c. of this chloroform solution in a small graduated test tube and fill to the 5 c.c. mark with chloroform.

In another test tube, place 5 c.c. of the cholesterol standard.

Add to both tubes 2 c.c. of anhydrous acetic acid and 5 drops of sulphuric acid, c.p. Mix and keep in the dark for 15 minutes.

Read in a colorimeter, setting the standard at 10.

Calculation: Divide 2,000 by the reading of the unknown to obtain the mg. of total cholesterol in 100 c.c. of whole blood.

Normal by this method is 140 to 190 mg. per 100 c.c.

Calculation of Total Fatty Acids: Subtract the total cholesterol from the total lipoids to obtain the total fatty acids.

Normal fatty acids by this method is 310 to 360 mg. per 100 c.c. of blood.

The Meyer and Schaeffer lipocytic coefficient is obtained by dividing the total cholesterol by the total fatty acids.

Normal is 0.45 to 0.52.

Evaporation Method.—

Carry out the evaporation through a glass tube conveniently bent so that it will bore through the two-holed stopper of the Erlenmeyer flask.

Through the other perforation of the stopper, place another glass tube, which leads from the Erlenmeyer flask and bores through the two-holed stopper of another container which is immersed in running water.

Another tube, bent and connected by a rubber tubing, leads from the second perforation of the last stopper; the rubber tubing empties into the suction at the sink.

In this way, one prevents igniting the ether-alcohol mixture on evaporation; it permits the introduction into the Erlenmeyer flask of the material in the water bath at any temperature without danger, and thus shortens the time of evaporation considerably; the ether-alcohol mixture used in the extraction can be recovered and later used for the remainder of the technic or for other purposes.

At the completion of this evaporation, separate the condenser, keeping the flask within the water bath at the level of the neck to prevent water of condensation in the neck. Keep it unstoppered in the boiling water bath for 5 minutes at sufficient temperature to assure a satisfactory dryness.

If other means of heating are used, it is very difficult to regulate, and there is also risk of spoiling the fatty blood extract obtained, which is affected by a temperature higher than 100° C.

Free and Total Cholesterol

Method of Zak, Dickenman, White, Burnett, and Cherney¹

Principle.—

The cholesterol is extracted in alcohol-acetone, dried, and the reaction is carried out in glacial acetic acid with an iron reagent. The resultant color obeys Beer's law.

Reagents.—

Alcohol-Acetone Mixture.—

Equal parts of absolute ethyl alcohol and acetone.

Digitonin Solution.—

Dissolve 1 gm. of digitonin in 50 c.c. of ethanol in a 100 c.c. volumetric flask.

Dilute to 100 c.c. with distilled water.

Iron Stock Solution.—

Dissolve 2.5 gm. of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 25 c.c. of glacial acetic acid.

Preserve in the freezing compartment of a refrigerator and thaw when needed. No precipitate forms in the thawed solution.

Color Reagent.—

Place 1 c.c. of the iron stock solution in a 100 c.c. volumetric flask and dilute to 100 c.c. with sulphuric acid, c.p., with continuous swirling. Discard when a precipitate forms.

Cholesterol Standard Solution.—

Dissolve 100 mg. of pure dry cholesterol in glacial acetic acid in a 100 c.c. volumetric flask, and dilute to 100 c.c. with acetic acid, mixing while diluting. 1 c.c. contains 1 mg. cholesterol.

Technic.—

Preparation of the Working Curve.—

Pipette 0.0, 0.1, 0.2, and 0.3 c.c., respectively, of cholesterol standard solution into 30 c.c. test tubes.

Dilute each to 3 c.c. with glacial acetic acid.

Add 2 c.c. of the color reagent to each, mix thoroughly, and allow to cool.

Measure the absorbance at 560 $\text{m}\mu$, using a 10 by 75 mm. cuvette.

Analysis of Specimen.—

Pour about 10 c.c. of a 50:50 alcohol-acetone mixture into a 25 c.c. volumetric flask.

Add 1 c.c. of serum with vigorous swirling and bring to a boil in a hot water bath, mixing constantly while heating to avoid bumping.

Cool, and dilute to the 25 c.c. mark with alcohol-acetone mixture.

Filter through Whatman 41-H filter paper, keeping the funnel top covered with a watchglass to prevent evaporation.

Place 2.5 c.c. aliquots respectively into a 30 c.c. test tube and a 15 c.c. conical centrifuge tube.

Place both tubes in a hot water bath.

Evaporate the contents of the test tube to dryness and of the conical tube to 0.5 to 1.0 c.c.

¹Zak et al.: Am. J. Clin. Path. 24: 11, 1954.

Total Cholesterol.—

Pipette 3 c.c. of glacial acetic acid into the test tube containing the dried residue and warm about 30 seconds in a water bath until the residue comes off the glass.

Add 2 c.c. of the color reagent and mix thoroughly.

Allow to cool.

Measure the absorbance at 560 $m\mu$, using a 10 by 75 mm. cuvette.

Free Cholesterol.—

Add 1 c.c. of a 1% digitonin solution to the partially evaporated contents of the centrifuge tube.

Wait 10 minutes, and centrifuge at 3,500 r.p.m. for 10 minutes.

Decant the supernatant fluid.

Invert the tube and drain for several minutes on absorbent paper.

Blow 4 c.c. of acetone into the tube to disperse the precipitate.

Mix thoroughly, and centrifuge at 3,500 r.p.m. for 10 minutes.

Again drain dry after decanting the supernatant fluid.

Pipette 3 c.c. of glacial acetic acid into the tube.

Warm a few seconds if necessary in a water bath to dissolve the precipitate.

Add 2 c.c. of the color reagent.

Mix, cool, and measure the absorbance at 560 $m\mu$ in a 10 by 75 mm. cuvette.

Calculation.—

Obtain the free and total cholesterol from the calibration curve.

Determination of Cholesterol, Leiboff

The method described by Leiboff¹ has the advantages over some previously reported methods in that it is less time-consuming and is more accurate since there is no loss of cholesterol possible during any of the operations.

Technic.—

All glassware, including pipettes, must be dried in an oven before using.

Put 5 c.c. of chloroform in the extraction tube (Fig. 117) and drop the filter paper disk (Fig. 118) into the extraction tube; it should lie at point X. The constricted portion of the tube thus serves a double purpose: it holds the paper disk in place, and permits accurate dilution.

Pipette 0.25 c.c. of oxalated blood on the filter paper disk. The blood is immediately absorbed by the filter paper. Then attach the extraction tube to a reflux condenser and immerse in a beaker of hot or boiling water placed over a small electric stove. The water in the beaker should be above the level of the chloroform in the tube.

Extract for 30 minutes. Detach the tube from the condenser, remove filter paper disk from tube, and cool by immersing in cold water for a minute. When cool, add chloroform exactly to the 5 c.c. mark.

In a similar extraction tube place 5 c.c. of a standard solution containing 0.4 mg. of cholesterol per 5 c.c. This standard is made by dissolving 40 mg. of pure dry cholesterol in 500 c.c. of pure dry chloroform.

Add 2 c.c. of pure dry acetic anhydride and 0.1 c.c. of concentrated sulphuric acid to each tube. Insert cork stoppers into the tubes, and invert twice to mix well. Place the tubes in a beaker of cold water for half a minute to cool, leave in a dark place for 10 minutes, and read in colorimeter.

Set the standard in the colorimeter at 10 or 15 mm., depending on the intensity of the color of the unknown.

Calculation.—

$$\frac{S}{R} \times 160 = \text{mg. cholesterol in 100 c.c. of blood.}$$

S = reading of standard.

R = reading of unknown.

¹Leiboff, S. L.: J. Biol. Chem. 61: 177, 1924.

Example.—

$$S = 15; R = 20.$$

$$\frac{15}{20} \times 160 = 120 \text{ mg. cholesterol in 100 c.c. blood.}$$

Normal is 150 to 300 mg. cholesterol in 100 c.c. blood.

Note.—An improvement in this method was subsequently reported by Leiboff.¹ This method uses straight pieces of filter paper without openings in the center. They are easily prepared from Whatman's double thickness extraction thimbles by cutting them into 19 mm. squares. These are just large enough to absorb 0.25 c.c. of blood, which is the required amount for the determination of cholesterol. The four corners of the paper are turned upward so that the squares fit in the extraction tubes. About 18 squares of paper are obtained from a 22 by 18 mm. extraction thimble. In order to allow the chloroform to pass freely without being obstructed by the filter paper, a side neck, connecting the upper and constricted portions, was added to the tube. The filter paper thus lies between the upper and lower openings of the side neck through which the chloroform passes without obstruction. This apparatus can be obtained from the Technicon Co., 215 E. 149th St., New York City.

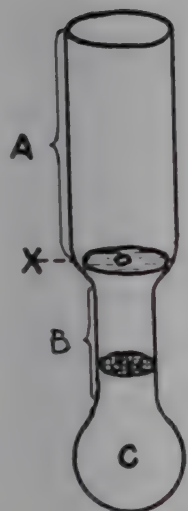


Fig. 117.



Fig. 118.



Fig. 119.—Leiboff apparatus for cholesterol estimation.

Cholesterol Method of Bloor (Modified)

Reagents.—

Stock Solution of Cholesterol.—(1 c.c. contains 1.0 mg. cholesterol.)

Add 0.2 gram of cholesterol (Merck) to 200 c.c. dry chloroform in a dry bottle.

Standard Solution of Cholesterol.—(For use) (1 c.c. contains 0.1 mg. cholesterol.)

Add 10 c.c. of stock cholesterol to 50 c.c. dry chloroform in a dry 100 c.c. volumetric flask.

Dilute to 100 c.c. with dry chloroform.

Acetic Anhydride.—

Redistilled Alcohol or Absolute Alcohol.—

Ether.—

Dry Chloroform.—(See Fatty Acids, above.)

Concentrated Sulphuric Acid.—

¹J. Lab. & Clin. Med. 11: 777, 1926.

Equipment.—

- 1 100 c.c. volumetric flask.
- 1 100 c.c. graduated cylinder.
- 2 3 c.c. volumetric pipettes.
- 2 2 c.c. volumetric pipettes
- 1 5 c.c. volumetric pipette.
- 1 10 c.c. volumetric pipette.
- 1 1 c.c. pipette graduated in 0.1 c.c.
- 2 10 c.c. glass stoppered tubes, graduated at 5 c.c.
- 1 4 inch funnel.
- 1 250 c.c. casserole.
- Filter paper.
- Electric hot plate and water-bath.

Principle.—

Cholesterol is extracted from blood filtrate by the use of chloroform, and the extract, after treatment with acetic anhydride and sulphuric acid, is compared with a standard solution of cholesterol similarly treated.

Technic.—

All glassware must be dried in the oven before use.

Prepare a mixture of 3 parts of absolute alcohol and 1 part of ether.

Place 80 c.c. of the ether-alcohol mixture in a 100 c.c. volumetric flask.

Add 3 c.c. whole blood, slowly, and with constant shaking of the flask.

Shake thoroughly.

Place in a water-bath over an electric hot plate.

Slowly raise the contents of the flask to boiling, frequently shaking the flask to prevent overheating.

Cool to room temperature.

Dilute to 100 c.c. with the mixture of ether and alcohol (above).

Mix thoroughly, and filter through filter paper.

The filtered liquid will keep in a tightly stoppered, dark bottle until the next day, if necessary, before completing the final determination.

Place 10 c.c. filtrate in a small beaker and evaporate just to dryness in the water bath. Take care not to heat beyond the point of dryness, as a brownish color is produced, which renders the determination difficult.

To this dry residue in the beaker add 2 c.c. dry chloroform.

Bring to a boil, and boil to half volume.

Decant, and place in a 10 c.c. glass-stoppered cylinder.

Add 2 c.c. more of chloroform and repeat the process.

Repeat several times, taking care not to increase the total volume to more than 5 c.c.

Cool the material in the cylinder and dilute to 5 c.c. with dry chloroform.

Develop the color of the solutions as follows:

Place in a glass-stoppered 10 c.c. cylinder marked "S" for standard 5 c.c. standard solution of cholesterol (1 c.c. = 0.1 mg.).

To both the unknown and the standard add 2 c.c. acetic anhydride and 0.2 c.c. concentrated sulphuric acid, c.p.

Mix, and let stand five to twenty minutes in a dark place.

Read in the colorimeter.

Make several readings, and take the average.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.5 \times \frac{100}{0.3} = \text{the mg. of cholesterol in 100 c.c. blood.}$$

Example.—

Reading of standard is 15; reading of unknown is 10; 0.3 represents the number of c.c. blood in the 10 c.c. filtrate used; 0.5 is the mg. of cholesterol in the standard solution.

$$\frac{15}{10} \times 0.5 \times \frac{100}{0.3} = 250 \text{ mg. cholesterol in 100 c.c. blood.}$$

Normal is 150 to 230 mg. cholesterol in 100 c.c. blood, for this method.

Method of Schoenheimer and Sperry for Free and Combined Cholesterol¹

Principle.—Cholesterol is precipitated by digitonin followed by the application of a color reaction to the precipitate. The isolation of cholesterol as digitonide avoids the errors which are introduced into other colorimetric methods by other chromogenic substances present in fatty extracts.

Reagents.—

Acetone-Absolute Alcohol (1:1).

Ether.—Wash this ether with a sodium sulphite solution followed by several washings with water. The washed ether is distilled over calcium chloride.

Acetone-Ether (1:2).

Digitonin Solution.—Dissolve 1 gm. of digitonin in one liter of distilled water. Place in a refrigerator at least 24 hours or longer and then centrifuge to pack the precipitate which forms. Digitonin used by these authors is made by Hoffmann-LaRoche, Inc. Filter supernatant fluid and concentrate to 500 c.c. This is done by placing in a weighed liter flask equipped with inlet and outlet tubes. Mouth of the inlet tube should be kept about 2 cm. above the surface of the solution. A rapid stream of air, filtered through cotton, is drawn by suction, through the flask which is immersed in boiling water. The point at which the required amount of water has been removed is determined with sufficient accuracy by weighing the flask with its contents on an ordinary laboratory balance. The concentration requires three to four hours. If a sediment appears, remove by filtration.

Potassium Hydroxide Solution made by dissolving 10 gm. of pure KOH in 20 c.c. of distilled water.

Hydrochloric Acid, Approximately 5 per cent, made by diluting 15 c.c. of concentrated HCl to 100 c.c. with distilled water.

Acetic Acid, 100 per cent. Eastman and Kahlbaum are equally good.

Acetic Anhydride. Use the product labeled "acetic anhydride 99-100 per cent" made by Eastman.

Concentrated Sulphuric Acid.

Apparatus.—

Volumetric flasks, 5 c.c.

Funnels, 2.5 cm. diameter.

Filter paper, 4.5 cm. diameter. Free paper completely of sterols with ether or hot alcohol.

Stirring rods, 13 cm. long.

Preserving jars, pint or quart size, with rubber gaskets.

Dropping bottles, with ground-in pipettes equipped with rubber bulbs.

Glass tube, 6 to 7 mm. in diameter, finely drawn out of capillary. The capillary tip should be 8 cm. long.

Transfer pipettes, for use with rubber bulbs. Make them by drawing out 8 mm. glass tubing. Length should be 13 cm., with tip 5 cm. long, with orifice large enough to permit rapid filling and emptying.

Water bath, equipped to hold 15 c.c. centrifuge tubes in the dark at 25° C. (Any pan of fairly large capacity, fitted with a rack and thermometer, is suitable. The pan may be placed in a box with a door or covered with a dark cloth to exclude the light.)

Zeiss Pulfrich photometer with 5 cm. microcells, or any standard photoelectric colorimeter.

¹Schoenheimer, R., and Sperry, W. M.: J. Biol. Chem. 106: 745, 1934.

Technic.—

Extraction of Serum or Whole Blood.—Place approximately 3 c.c. of the acetone-alcohol solution in a 5 c.c. volumetric flask and bring to boil on steam bath.

Add 0.2 c.c. serum or whole blood, slowly, with capillary pipette with shaking. Insert stopper and shake vigorously. Remove stopper, wash, and heat contents of flask to boiling.

Cool to room temperature. Make up to volume with alcohol-acetone, mix, and filter through small dry filter. Filtrate should be clear.

Precipitation of Free Cholesterol.—Pipette 2 c.c. of filtrate into 15 c.c. centrifuge tube, add 1 c.c. of digitonin, stir with rod and leave the rod in the tube. Place in preserving jar. Cover tightly. Leave overnight at room temperature (one hour's standing is usually sufficient). Place tube in test tube rack and stir gently to free particles that adhere to tube. Remove stirring rod carefully. Place rod carefully on wire rack so as not to lose any particles that may adhere to it. Centrifuge tube at 2,500 r.p.m. for 15 minutes. Remove supernatant fluid with capillary pipette, using suction pump cautiously. Do not touch the wall of the centrifuge tube with the pipette or stir up precipitate. A few particles, which are cholesterol ester, float and are drawn off with the solution.

Replace stirring rod in tube and wash down the rod and wall of tube with 1.5 to 2.0 c.c. of the acetone-ether solution. Add wash solution from dropping pipette with a rubber bulb.

Stir precipitate, remove rod from rack, centrifuge tube for 5 minutes. Remove supernatant solution as before. Use less suction because this acetone-alcohol solution is more easily drawn off. Wash precipitate twice more, using ether instead of acetone-ether. Place stirring rod in tube; place in water bath at 40° C. Precipitate dries in 2 to 3 minutes. Remove last traces of ether by holding a tube attached to a suction pump in the test tube for a few minutes. Sample is ready for color development.

Precipitation of Total Cholesterol.—Pipette 1 c.c. of extract into a 15 c.c. centrifuge tube. Add one drop of KOH solution and stir with stirring rod, leaving the rod in the tube. Place in preserving jar containing a layer of sand about 3 cm. deep heated to 40° C. Sand acts as heat reservoir. Clamp on cover tightly and keep at 37° to 40° C. for half hour.

Cool tube after this hydrolysis, add 1 c.c. acetone-alcohol, titrate with 5 per cent HCl (phenolphthalein indicator). Stir constantly and be sure to get excess of acid.

Add 1 c.c. digitonin, stir constantly, allow tube to stand one hour or longer, then centrifuge precipitate 5 minutes and wash as before.

Development and Reading of Color.—Dissolve the dried precipitate of digitonide in 1 c.c. acetic acid. Wash down walls in adding acid. Hasten solution by warming the tube to 60° C. and stirring.

Adjust temperature of water bath to 25° C.; place tubes in bath for a few minutes. Remove one tube, place in rack in a small pan or beaker, containing water at 25° C. Add 2 c.c. acetic anhydride, followed by 0.1 c.c. concentrated sulphuric acid added from automatic microburet. Stir with stirring rod that was left in the tube and replace in water bath.

Remove another tube to the water bath and add reagents as before. Interval between the reagents is so timed that not less than 27 minutes nor more than 37 minutes elapse between the addition of the sulphuric acid and the reading.

In reading the color with the photometer, fill one cell with a blank (1 c.c. acetic acid, 2 c.c. acetic anhydride, 0.1 c.c. sulphuric acid); other cell with unknown, using a transfer pipette. Reverse cells after reading and take reading on the opposite side.

If an ordinary colorimeter is used, compare unknown with a standard solution of anhydrous cholesterol in acetic acid. Time for color development for unknown and standard should be the same.

The calculation of the amount of cholesterol is based on the results of a long series of determinations on known amounts of cholesterol (see Table 23).

$E_{sp.} = E/\text{No. of mg. of cholesterol}$; $E = (-\log [T/100])/L$ (where T is the percentage transmission and L the length of the cell in cm.). The value of $E_{sp.}$, to be determined by the operator, was found by these workers to be 1.450. The amount of cholesterol is given by the value, $E_{obs.}/E_{sp.}$

TABLE 23.—SPECIFIC EXTINCTION COEFFICIENTS OF DIFFERENT AMOUNTS OF CHOLESTEROL PRECIPITATED ACCORDING TO PROCEDURE DESCRIBED

(From Schoenheimer and Sperry)

AMOUNT (MG.)	NO. OF DETER- MINATIONS	AVERAGE E_{sp} .	STANDARD DEVIATION	PROBABLE ERROR*
0.15	15	1.447	± 0.0111	± 0.0019
0.10	18	1.454	± 0.0110	± 0.0017
0.05	20	1.449	± 0.0226	± 0.0034
0.04	11	1.454	± 0.0115	± 0.0023
0.03	12	1.447	± 0.0226	± 0.0044
Average-----		1.450	± 0.0174	± 0.0013

*With 0.025 mg. the probable error was found to be somewhat larger than the values shown. Very little color is produced by such small amounts, and the error in reading is correspondingly great. Amounts smaller than 0.03 mg. are rarely met with in working with serum or blood.

LaMotte Blood Cholesterol Outfit

(Micromethod)

This is a micromethod for total cholesterol, based on the modified Bloor alcohol-ether extraction procedure. Cholesterol is extracted from the blood with an alcohol-ether mixture, and the cholesterol is determined colorimetrically by the simple comparator method. Only 0.2 c.c. of blood is required for a test and the test can be completed in less than one hour. This test is excellent for office practice, since only the simple equipment contained in the outfit is required for the test.

Interpretation of Cholesterol Findings in Blood

Considerable attention has been given to the subject of cholesterol and its diagnostic importance. A few facts might first be stated as to just what cholesterol is: It is a monatomic, simple, unsaturated, secondary alcohol. It is a substance found throughout the human organism and is a constituent of various animal foods. Fraser and Gardner¹ state that the phytosterols of the plant foods are transformed to cholesterol in the body.

It is a disputed question whether or not cholesterol is synthesized in the body. Lifschütz² thinks that it is formed from oleic acid and also that it holds some relationship to cholic acid³ since the same color reactions are obtained after oxidation with benzol peroxide. Goodman⁴ found that cholesterol injected directly into the circulation appears to have but slight influence on the elimination of cholic acid. Rosenbloom and Gies⁵ suggest that gallstones may arise, when among other causes the transformation into bile is materially diminished with a subsequent marked increase in the concentration of cholesterol in the bile.

Cholesterol occurs in the blood in the free and the combined state. Free cholesterol occurs in the corpuscles and to some extent in the plasma, and the cholesterol esters in the plasma alone. Bloor and Knudson⁶ found in the whole blood the average percentage of cholesterol in combination in esters was about 33.5 per cent and in the plasma 58 per cent of the total cholesterol. Normally the concentration of cholesterol is the same in plasma and whole

¹Fraser and Gardner: Proc. Roy. Soc., London (B) 82: 559, 1910.

²Lifschütz: Ztschr. f. physiol. Chem. 55: 1, 1908.

³Lifschütz: Ztschr. f. physiol. Chem. 92: 383, 1914.

⁴Goodman: Beitr. z. chem. phys. u. Path. 9: 91, 1907.

⁵Rosenbloom and Gies: Biochem. Bull. 1: 51, 1911-1912.

⁶Bloor and Knudson: J. Biochem. 29: 7, 1916.

blood. The average found by Bloor was 0.21 per cent for normal men and 0.23 per cent for normal women. Gorham and Myers⁷ state that the figures of Bloor are too high and that possibly 0.16 or 0.17 per cent may more nearly represent the true value for the cholesterol of human blood. Gardner and Gainsborough⁸ give the normal value as 169 and 153 mg. per hundred cubic centimeters of plasma for men and women, respectively, with standard deviations, respectively, of 41 and 33 mg. per hundred cubic centimeters. Quoting from a later review by Weinhouse,⁹ "more recent studies have established high normal values as well as a wide range of normal variation. Kirk, Page, Lewis, Thompson, and Van Slyke, in 1935, found that the concentration of total cholesterol in plasma varied from 109 to 376 mg. per hundred cubic centimeters, with a standard deviation of 62 mg. . . . In its variability, cholesterol differs significantly from most of the other constituents of the blood, which normally fluctuate between narrow limits."

So far as the factor of age is concerned, Weinhouse⁹ states that the constancy of the ratio of free to total cholesterol, observed in adults, is not characteristic of infants' plasma, in which the amount of combined cholesterol in the total varies from 41 to 72 per cent. Further he states that there is a gradual and slight increase with age throughout childhood. In a study of normal men, 21 to 91 years of age, Kirk and co-workers¹⁰ found no significant effect of age on the levels of cholesterol and its esters or other lipids in the blood plasma, nor any change in their proportions.

What are the changes in pregnancy? Weinhouse⁹ states that during the first trimester there is no change. Gradual rise in all the lipids begins in the fourth month and continues up to the eighth month. It remains high during the puerperium and then declines to normal.

Effect of Diet.—There is considerable difference of opinion on this point. While Gardner and Gainsborough reported that there was a change in the amount in the blood dependent upon the amount in the diet, Turner and Steiner observed nine patients for many months, and concluded that no relation existed between the type of diet and the blood cholesterol value.

Summary of Normal Values.—Weinhouse summarizes from all the available literature that the concentration of cholesterol in the serum of normal human beings of both sexes averages about 200 mg., with a range of from 100 to 400 mg. Long periods of fasting produce no change in the blood levels. "Apparently in man hypercholesteremia does not develop as a result of the ingestion of moderate quantities of cholesterol."

Pathologically a great many conditions have been recorded in which a hypercholesterolemia was found, for instance, arteriosclerosis, nephritis, diabetes, especially with acidosis, obstructive jaundice, in many cases of cholelithiasis, in the early stages of malignant tumors, and in pregnancy.

Gorham and Myers made cholesterol estimations in the blood of about 200 subjects suffering clinically from twenty-five different diseases. Hypercholesterolemia was observed, though not invariably, in arteriosclerosis, nephri-

⁷Gorham and Myers: *Arch. Int. Med.* 4: 599, 1917.

⁸Gardner, J. A., and Gainsborough, H.: *Biochem. J.* 21: 130, 1927.

⁹Weinhouse, Sidney: *The Blood Cholesterol*, *Arch. Path.* 35: 438-501, 1943.

¹⁰Kirk, E., Page, E. H., and Van Slyke, D. D.: *J. Biol. Chem.* 3: 613, 1935.

tis, obstructive jaundice, and diabetes. A hypercholesterolemia was observed in the cachexia of malignancy and all anemias of the pernicious type. The cholesterol values encountered in the blood plasma of patients with pernicious anemia were regarded by these observers as of considerable significance, especially in view of the strong antihemolytic action of cholesterol.

Weinhouse* states that the more recent studies agree that in parenchymatous hepatic disease the serum cholesterol esters reach low levels. Lowered levels of blood cholesterol esters were especially marked in acute, subacute, and chronic yellow atrophy. Further, this reviewer states that "in contrast to jaundice from damage of the liver, the jaundice associated with biliary obstruction is accompanied by hypercholesteremia in 78 per cent of patients in whom the condition was verified at operation or at autopsy. There was no marked correlation between the blood cholesterol and the icteric index, although with relief of the obstruction and diminution of the jaundice, the cholesterol was reduced to normal." Further, he states that at present the explanation of the marked reduction of blood cholesterol esters in hepatic disease points to the inability of the hepatic cells to synthesize cholesterol esters or to transport them into the blood.

On normal subjects, fourteen in all, taken at random and not on a special diet, the figures of Gorham and Myers varied from 0.13 to 0.19 per cent. Their average was 0.15 per cent which compares very well with the data already in the literature. In ten cases of arteriosclerosis, the figures ran from 0.16 to 0.26 per cent, showing hypercholesterolemia. These figures compare well with those already given by Schmidt.¹ Weinhouse* records that in many, if not most, cases of arteriosclerosis, there is no accompanying increase in cholesterol. "Although the relation of the blood lipids to the development of arteriosclerosis remains undisclosed, there is hardly any doubt that the disease can develop in the absence of any abnormality in the levels of the blood lipids." He very properly makes the point that before conclusions can be drawn regarding the influence of hypercholesteremia on the development of arteriosclerosis in human beings, it is necessary to know the incidence of arteriosclerosis in diseases accompanied by hypercholesteremia, such as hypothyroidism, nephrosis, and hepatic diseases. At present, there is no convincing evidence that arteriosclerosis results from hypercholesteremia alone.

In the figures of Gorham and Myers on nephritis, while the percentages were increased, there was no apparent relation between the cholesterol in the blood and the blood pressure or nitrogen retention. Excepting the observation of Denis² who found the cholesterol increased in only one case of nephritis out of a very large series, the observations of Gorham and Myers agree with those already in the literature. In eight cases of diabetes they found an increase in cholesterol in the blood of but four cases, and these four cases showed evidences of acidosis. Since, as has been pointed out by Bloor,³ the cholesterol increases along with the other lipoids in diabetic lipemia, the cholesterol may be taken as an index of the lipid content of the blood.

*Loc. cit.

¹Schmidt: *Arch. Int. Med.* 13: 121, 1914.

²Denis: *J. Biol. Chem.* 29: 93, 1917.

³Bloor: *J. Biol. Chem.* 26: 417, 1916.

A survey of the quantitative studies of lipids in Bright's disease, states Weinhouse, reveals that high blood cholesterol is characteristic of true nephrosis and the chronic nephrotic stage of nephritis. Page, Kirk, and Van Slyke found that the chronic stage of hemorrhagic nephritis was accompanied by hyperlipemia.

The work of Rothschild and Felsen and their conclusions are not at all in accord with the above observations of Gorham and Myers. They had previously shown that a number of patients with cholelithiasis had a continuous hypercholesterolemia. Even after operation at which all causes for an obstructive hypercholesterolemia had been removed, these patients again became hypercholesterolemic with no discoverable basis for the condition. They believe that the liver is the regulator of the cholesterol metabolism of the body, the cholesterol being kept at a more or less constant level by excretion of cholesterol through the bile. In a later communication these observers contend that in obstructive icterus due to stones, the cholesterol content of the blood is markedly elevated and bears a definite relationship to the intensity of the icterus. They also conclude that, in conditions associated with hepatic disorders, the cholesterol content of the blood is not increased, and is usually reduced. The cholesterolemia is not proportionate to the amount of bile pigments present in the blood. In the so-called hemolytic icterus they found no increase of blood cholesterol. They found in obstructive jaundice amounts of cholesterol as high as 700 mg. per 100 c.c. of blood. They found that a patient with jaundice and high temperature and an infection will have a lower cholesterol content than a patient with the same degree of jaundice, but with no active infection. It is interesting to note that these observers found the blood low in cholesterol in three cases of acute yellow atrophy of the liver.

In hyperthyroidism and hypothyroidism: Weinhouse indicates in his review that subnormal values are to be expected to accompany hyperthyroidism. "In hypothyroidism, the variations from normal cholesterol values are large, and small changes in the metabolic rate effect relatively great changes in the cholesterol level." Blood chemical studies therefore are useful in the diagnosis and treatment of such cases. Boyd and Connell¹ have shown that the hypercholesteremia of myxedema is part of general hyperlipemia.

In xanthomatosis, the deposits show a high lipid content. Cholesterol is a major constituent. Weinhouse concludes that although secondary xanthomatosis probably results from an increase in the quantity of cholesterol in the serum or from a disturbance in the colloidal state of the lipid complex, primary essential xanthomatosis may occur without obvious abnormality of the blood lipids.

One must note Weinhouse's general attitude of skepticism concerning the reliability of the usual clinical laboratory tests for these substances, cholesterol and its esters. He advances two serious objections to direct colorimetric determinations: first, an appreciable difference exists between free and bound cholesterol in the amount and velocity of the color development; second, it has been shown that cholesterol esters yield values ten to thirty per cent higher

¹Boyd, E. M., and Connell, W. F.: *Arch. Int. Med.* 61: 755, 1938; *Quart. J. Med.* 5: 455, 1936.

than the equivalent amounts of free cholesterol. Another source of error in the direct colorimetric procedure is the presence of interfering colors in the lipid extract and the possibility that other substances besides cholesterol in blood may contribute to the color (Reinhold). Weinhouse recommends for accurate work a method based upon precipitation with digitonin, notably, the method of Schoenheimer and Sperry.* (See page 367.)

Further, Weinhouse issues a word or two of caution in interpretation by having the consultant consider whether the determination was carried out on serum, plasma, or whole blood. Values for whole blood will be characterized by a lower level for the total free cholesterol and an increased proportion of free cholesterol. Shope found lower cholesterol values for oxalated or citrated plasma than for serum. Serum and heparinized plasma give identical values for free and total cholesterol, which more truly represent their concentrations in the noncellular portions of the blood.

A query on the normal values of cholesterol using both serum and plasma appeared in the *Journal of the American Medical Association*.† It was answered as follows in this publication:

“It has been generally accepted that the total cholesterol concentration of the serum and of the plasma is the same, but normal values for the Bloor method should not be given without some explanation.”

Grigaut, who presented the first colorimetric method of estimating cholesterol in the blood with the Liebermann-Burchard color reaction¹ in 1913 presented data on simultaneous analyses of serum and plasma² in which practically identical findings were found in serum and in plasma. It would not appear that this observation has been questioned, since some workers employ plasma while others use serum.

The Autenreith and Funk method,³ which Bloor and many early workers in this country employed, was simply an adaptation of the Grigaut method for use with the Hellige colorimeter. Although Bloor, when he first adapted this method to his alcohol-ether extract of blood, saponified the extract with sodium ethylate,⁴ he later omitted the saponification when he found that the cholesterol esters also gave the color reaction.⁵ He observed that the results with the simplified method averaged 20 per cent higher than with the older procedure in which saponification was employed. Bloor suggested, as a possible explanation for the lower values obtained, the action of the strong alkali when saponification was employed. Many other explanations for this difference have been suggested by different workers. However, it remained for Gardner and Williams⁶ to note that esterified cholesterol reacts at a greater rate in development of color than does free cholesterol. Kelsey, in Bloor's laboratory, has

*Schoenheimer, R., and Sperry, W. M.: *J. Biol. Chem.* **106**: 747, 1934.

†*J. A. M. A.* **127**: 15, 1024, April 14, 1945.

¹*Compt. rend. Soc. de biol.* **68**: 791, 1910.

²Myers, V. C.: *Practical Chemical Analysis of Blood*, ed. 2, St. Louis, 1924, The C. V. Mosby Company, p. 124.

³*München, med. Wchnschr.* **60**: 1243, 1913.

⁴*J. Biol. Chem.* **23**: 320, 1915.

⁵*Ibid.* **24**: 227, 1916.

⁶*Biochem. J.* **15**: 371, 1921.

verified this observation¹ and has shown that cholesterol palmitate gives 30 per cent more color than does free cholesterol. Since Sperry² has shown that the total cholesterol in the normal human serum averages 73.1 per cent cholesterol esters with a relatively constant ratio, this explains why the results with the commonly used Bloor (1916) method are about 20 per cent too high for total cholesterol.

Bloor obtained a range of values for total cholesterol of 190 to 310 mg. per 100 c.c. of plasma for normal adults, with an average of 220 mg. for males and 240 mg. for females. This would be equivalent to a range of about 150 to 250 mg. for true cholesterol.

BLOOD DIASTASE TEST AND ITS INTERPRETATION

Attempts have been made for a long time to measure the external secretory function of the pancreas as an aid in the diagnosis of diseases affecting the acinar tissue of the pancreas. R. Elman³ has given an extensive description of the various tests used in the determination of this function. As set forth by Cole and Elman,⁴ the blood diastase has been studied with a view of determination of changes in pancreatic secretion. Of the three enzymes in the pancreatic juice, diastase has received the greatest attention. It is found by these writers that a method modified by Somogyi is the simplest to perform as a routine in most laboratories.

Micromethods for the Estimation of Diastase (Amylase)

Method of Somogyi

Somogyi⁵ found that the existing micromethods devised for the purpose of estimation of diastase are inadequate for quantitative measurements. Pre-existing methods lack standardization of important factors such as hydrogen ion concentration and electrolyte content, which are known seriously to influence diastatic activity. Some of the methods disregarded the establishment of the proper relationships between enzyme activity and the amount of substrate.

Somogyi gives two micromethods for the estimation of diastatic activity in blood, urine, and other biologic material. These methods are quantitative in the sense that the quantities that are measured are in linear proportionality with the diastatic activity. In the first method the saccharogenic action of the enzyme is determined with due regard to available information concerning the kinetics of the reaction and to necessary precautions in the analytical technic involved.⁶

The second method, which is based upon the amyloclastic action of diastase, was prompted by two objectives. In the first place, a technic, simpler and more rapid than the estimation of reducing power, seemed desirable in view of the clinical significance of diastase determinations. Secondly, it was deemed advisable by Somogyi to check and control the results of his copper reduction

¹J. Biol. Chem. **127**: 15, 1939.

²Ibid. **114**: 125, 1936.

³In Graham's Surgical Diagnosis, Vol. II, Philadelphia, 1930, W. B. Saunders Company, p. 550.

⁴Textbook of General Surgery, New York, 1936, D. Appleton-Century Company, p. 600.

⁵Somogyi, Michael: J. Biol. Chem. **125**: 399, 1938.

⁶Somogyi, M.: J. Biol. Chem. **124**: 179, 1938.

method by another method which is built on a basically different analytical procedure. Both procedures are served only if the measurement of the amylolastic action yields results which parallel those obtained in the determination of the saccharogenic action of the enzyme. Observations of Johnson³ and of Sherman, et al., point to the existence, under certain conditions, of such parallelism; Somogyi's task, which he accomplished, was to establish these conditions for a microtechnic.

Principle.—

The amylase activity is determined on blood plasma or serum by determining the reducing sugars formed by the action of plasma or serum on starch at pH 7.2. In other words, 1 mg. of reducing sugar equals 1 unit of amylase.

For this test, blood should be drawn at the height of the attack of acute pancreatitis and the amylase determined a short time after withdrawing the blood.

If the values are above 400 units, dilute the serum or plasma with 5% sodium chloride solution before incubation.

The following sources of error must be taken into account: (1) incorrect temperature of the water bath; (2) incorrect timing of the incubation period; (3) the same errors as occur in blood sugar determinations.

Equipment.—

- 1 medium size test tube.
- 1 2 c.c. volumetric pipette.
- 3 1 c.c. graduated pipettes.
- 1 2 inch funnel.
- 2 1 × 8 inch test tubes.
- 3 5 c.c. volumetric pipettes.
- 1 buret.
- 1 receptacle for collecting filtrate.
- Small filter paper.

Method 1 (based on the estimation of saccharogenic activity).—

Reagents.—

Starch Paste.—Use U.S.P. cornstarch or pure rice starch.

Wash the starch as follows: Suspend 100 gm. of starch in 1 liter of approximately 0.01 N hydrochloric acid and agitate frequently for about an hour. After sedimentation, pour the acid off and stir up the starch in 1 liter of approximately 0.05 per cent sodium chloride solution. After sedimentation and decantation, repeat once more the washing with salt solution; then spread the starch out and allow it to dry in the air.

Prepare a paste with approximately 15 gm. of washed starch per liter of water. Thoroughly grind the starch in a mortar with 50 c.c. of water, while 900 c.c. of water are heated to boiling. Transfer the ground starch suspension into the hot water with vigorous agitation, using 50 c.c. of water to rinse the mortar. After boiling for one-half to one minute (with agitation), heat the starch paste in a water bath at 40° C. for 15 to 30 minutes. Keep the mouth of the flask covered by an inverted beaker during the heating period. Grinding exerts the favorable effect of keeping the starch in a well-dispersed state for a long time, whereas without the grinding it soon forms a heavy sediment.

Acid Sodium Chloride Solution.—

Place 10 gm. of sodium chloride, c. p., in a liter volumetric flask.

Dissolve in a small amount of distilled water.

Add 3 c.c. of N/10 hydrochloric acid.

Dilute to 1000 c.c. with distilled water. The presence of the acid is necessary for correction of the pH of blood serum or plasma which tends to rise above the upper limit of the optimum pH range (7.0 to 7.4) owing to loss of CO₂ during the preparatory manipulations.

³Johnson, W. A.: J. Am. Chem. Soc. 30: 798, 1908.

Protein Precipitants.—

5% Copper Sulphate Solution (CuSO₄.5H₂O).—

Dissolve 5 gm. copper sulphate, c. p., in enough distilled water to make 100 c.c.

6% Sodium Tungstate Solution.—

Dissolve 6 gm. sodium tungstate, c. p., in enough distilled water to make 100 c.c.

7% Copper Sulphate Solution.—

Dissolve 7 gm. copper sulphate, c. p., in enough distilled water to make 100 c.c.

10% Sodium Tungstate Solution.—

Dissolve 10 gm. sodium tungstate, c. p., in enough distilled water to make 100 c.c. Use the 5% copper sulphate and the 6% sodium tungstate for the deproteinization of plasma or serum. For whole blood, use the 7% copper sulphate and the 10% sodium tungstate.⁴

Copper Reagent of High Alkalinity (Shaffer-Somogyi).—

This reagent, used for the determination of reducing sugars, is similar to the one described by Somogyi in 1937 and is prepared according to directions given then,⁵ with the one difference that Na₂SO₄ is left out from among its constituents. The reason for this omission is that the filtrates to be analyzed in this procedure contain appreciable quantities of undigested starch, which, in the course of the sugar determination, coagulates in the presence of sodium sulphate and interferes with the iodo-metric determination of the reduced copper.

- Sodium carbonate -----25 gm.
- Rochelle salt -----25 gm.
- N/1 sodium hydroxide -----40 c.c.
- Copper sulphate (CuSO₄.5H₂O) ----- 6 gm.
- Potassium iodide ----- 5 gm.
- Potassium iodate, N/1 -----15 c.c.
- Dissolve in enough distilled water to make 1000 c.c. of solution.

TABLE 24.—GLUCOSE PER 100 C.C. OF SOLUTION CORRESPONDING TO TITRATION VALUES WHEN 5 C.C. OF 1:10 SOLUTION AND 5 C.C. OF HIGH ALKALINITY COPPER REAGENT ARE HEATED IN WATER BATH FOR 20 MINUTES

C.C. OF 0.005 N THIO- SULPHATE	TENTHS OF 1 C.C. OF 0.005 N SODIUM THIOSULPHATE									
	0	1	2	3	4	5	6	7	8	9
	MG. OF GLUCOSE IN 100 C.C. OF SOLUTION									
0					18	22	26	29	32	35
1	39	42	45	48	52	55	58	61	65	68
2	71	74	78	81	84	87	90	93	96	99
3	102	106	109	112	115	118	121	124	127	130
4	133	137	140	143	146	149	152	155	158	161
5	164	168	171	174	177	180	183	186	189	192
6	195	199	202	205	208	211	214	217	220	223
7	226	229	232	235	238	241	244	247	250	253
8	256	260	263	266	269	272	275	278	281	284
9	287	290	293	296	299	302	305	308	311	314
10	317	321	324	327	330	333	336	339	342	345
11	348	352	355	358	361	364	367	370	373	376
12	379	383	386	389	392	395	398	401	404	407
13	410	414	417	420	423	426	429	432	435	438
14	441	445	448	451	454	457	460	463	466	489
15	472	475	478	481	484	487	490	493	496	499
16	502	506	509	512	515	518				

This table shows the glucose equivalents of reduction values obtained with this reagent.

⁴Somogyi, M.: J. Biol. Chem. 90: 725, 1931.

⁵Somogyi, M.: J. Biol. Chem. 119: 741, 1937.

Technic.—

Into a test tube of 14 to 16 mm. diameter, labeled "U," pipette 5 c.c. of cornstarch paste.

Add 2 c.c. of acid sodium chloride solution.

Mix well and immerse in water bath at 40° C.

After a few minutes, when the fluid has assumed the temperature of the water bath, add 1 c.c. of serum or plasma.

Stopper and invert a few times.

Incubate for 30 minutes. At this point the action of the enzyme is terminated in conjunction with the deproteinization of the unknown.

To precipitate the plasma protein after thirty minutes' incubation, add 1 c.c. of 5% copper sulphate solution and 1 c.c. of 6% sodium tungstate solution.

Shake well and centrifuge 5 to 10 minutes at 1000 r.p.m. Since copper salts inhibit but do not fully stop diastase action, these operations must be carried out without undue delay.

Filter through small funnel and filter paper, collecting all of the filtrate.

This filtrate is enzyme-free. The filtrate, a 1:10 dilution of the plasma, is opalescent because of the presence of unaltered starch and is ready for the determination of its copper-reducing power.

Pipette 5 c.c. of the filtrate thus obtained to a large test tube labeled "test."⁶

Add 5 c.c. Shaffer-Somogyi high alkalinity copper reagent.

Cover and place in a boiling water bath for 20 minutes.

Run a blank determination along with the unknown and control. (See below.)

Cool 2 or 3 minutes under running tap water.

Add 5 c.c. N/1 sulphuric acid or 1 c.c. of 5 N sulphuric acid.

Mix well to dissolve completely the cuprous iodide which sometimes precipitates, especially when the reduction is considerable or if the Cu_2O has settled from long standing before titration. Allow to stand, covered, 5 to 10 minutes, with occasional agitation so long as undissolved cuprous oxide or iodide is visible (important). Rinse the cover and walls.

Titrate with 0.005 N sodium thiosulphate (pages 27 and 234). The end point may be set with split drops.

If the titration is less than 1 c.c., the result is doubtful because of approach to the capacity of the reagent. The determination must be repeated with a greater dilution of the sugar solution.

Control (Blood Sugar).—

Dilute 1 c.c. of plasma or serum with 7 c.c. distilled water.

Add 1 c.c. of 5% copper sulphate solution.

Add 1 c.c. 6% sodium tungstate solution.

Filter, using small funnel and filter paper, and determine reduction as in above procedure using 5 c.c. of the filtrate.

Add 5 c.c. Shaffer-Somogyi high alkalinity copper reagent.

Cover and place in boiling water bath 20 minutes.

Cool 2 or 3 minutes under running tap water.

Add 5 c.c. N/1 sulphuric acid or 1 c.c. 5 N sulphuric acid.

Titrate with 0.005 N sodium thiosulphate adding starch paste as an indicator.

Blank.—

A blank titration must be made with this test as with any iodometric method.

The amount of 0.005 N sodium thiosulphate required to combine with the free iodine in 5 c.c. aliquot of the high alkalinity copper reagent is determined in the blank. In the unknown and the control (blood sugar) some of the iodine is bound by the copper-glucose reaction and the excess free iodine is titrated with 0.005 N sodium thiosulphate.

⁶Shaffer, P. A., and Somogyi, M.: J. Biol. Chem. 100: 695, 1933.

Place in a large test tube 5 c.c. distilled water.

Add 5 c.c. Shaffer-Somogyi high alkalinity copper reagent.

Heat in the boiling water bath at the same time as the tubes containing the test and the control.

Cool and add 5 c.c. of N/1 sulphuric acid or 1 c.c. of 5N sulphuric acid.

Titrate with 0.005 N sodium thiosulphate, adding starch paste as an indicator. Record the number of c.c. used.

Calculation.—

Subtract the titration results in c.c. of both the control (blood sugar) and the unknown from the result of the blank titration.

Use the resulting figures to read the sugar values directly from Table 24, page 376.

Subtract glucose content of the plasma (control) from the glucose value of the unknown.

The difference represents the reducing matter formed by the diastase (amylase).

Example.—

Titration of blank was 15. Titration of unknown was 8.2. $15 - 8.2 = 6.8$. Sugar value read directly from Table 24 is 220 mg. of glucose per 100 c.c. of serum or plasma.

Titration of blank was 15. Titration of control was 12.1. $15 - 12.1 = 2.9$. Sugar value read directly from Table 24 is 99 mg. of glucose per 100 c.c. of serum or plasma.

$220 - 99 = 121$, the reducing matter formed by the amylase. This means that 100 c.c. of plasma or serum under the standardized conditions described above produce a quantity of reducing matter (glucose) which, in regard to reducing capacity, is equivalent to 121 mg. of glucose. This figure is designated as the diastase (amylase) value of the blood plasma or serum.

Normal.—The diastase value of normal human plasma ranges from 80 to 150, with an average of 115. In a small number (less than 10 per cent) of normal human beings, values between 60 and 80 are found in the lower range and up to 200 in the higher.

Note.—On acidification, the liberated iodine gives a strong blue color with the excess starch that has been carried over into the filtrate. This dark color makes it difficult to observe the point where the white cuprous iodide has gone completely into solution. To avoid errors on this account it is well to agitate the fluid frequently and long enough before titration with thiosulphate, in order to enhance oxidation and dissolution of the iodide. Again, at the titration, special care must be exercised shortly before the end point is reached, since the undigested starch present, which is considerable in amount, in particular when the diastatic activity had been low, tends to hold the last remnant of free iodine so firmly adsorbed that the blue starch-iodine complex may persist for a brief period in the presence of excess thiosulphate, and overtitration may be the consequence. In order to avoid such error, it is advisable to stopper and shake the contents of the test tube shortly before the titration is completed.

Method 2 (based upon color reaction with iodine).—

The amyloclastic activity of blood plasma is represented by the expression, $1/(t \times v)$, in which t = reaction time in minutes, and v = volume of plasma in c.c. Provided that the amyloclastic action of plasma, as determined by this technic, runs parallel with the diastatic activity, D , as determined by the copper reduction method, then $D = K \times 1/(t \times v)$, K being a constant that can be calculated if both D and t are determined on a series of plasma specimens. Figures accumulated on hundreds of samples show K actually to represent a constant of an approximate value of 1600, with some deviations due in the main to the variability in the extent of deterioration of the enzyme during incubation.

Reagents.—

Starch Solution.—

Use the same washed cornstarch as is used in the copper reduction method.

In the first step, rub up 15 to 20 gm. of starch in a mortar with 100 c.c. of 0.25% sodium chloride solution.

Pour the suspension, with vigorous agitation, into 900 c.c. of boiling 0.25% sodium chloride solution.

Boil for 1 minute, with continuous stirring.

Allow the *covered* solution to stand at room temperature for a day or longer if necessary. The greater part of the starch separates out, forming a sediment. The limpid supernatant fluid is to be used.

Syphon off or centrifuge and remove the supernatant fluid.

Sterilize by immersing the flask in a boiling water bath for 30 minutes, covering the mouth of the flask with an inverted beaker.

This is the *stock solution*, which, when properly diluted, furnishes the substrate.

To find the extent of dilution, the starch content (usually 0.4 to 0.7%) of the stock solution is determined as follows:

Introduce 5 c.c. of this starch solution into a large test tube (25 by 200 mm.).

Add 1 c.c. of approximately 5 N sulphuric acid.

Cover the test tube with a small beaker and heat submerged in a boiling water bath for 3 hours. Cool.

Add 1 drop of phenolphthalein indicator.

Neutralize with sodium hydroxide solution.

Dilute to 100 c.c. with distilled water.

Determine the glucose content.

Multiply this by 0.9 to obtain the starch content of the stock solution.

This serves as the basis for dilution.

Example.—If the concentration of starch was found to be 0.428%, measure 75 c.c. of the solution into a 500 c.c. graduated cylinder, and dilute with 0.25% sodium chloride solution to 428 c.c. The diluted solution then contains 75 mg. of starch and 250 mg. of sodium chloride in 100 c.c.

The reagent must be kept sterile, since the slightest contamination with microorganisms destroys its usefulness. At the same time, however, it must be accessible to daily use. Somogyi satisfied both requirements by keeping the reagent in a flask which is closed with a 2-hole rubber stopper, one hole holding a syphon tube, and the other an air inlet tube. The latter is filled with sterile cotton. When preparing the syphon flask, Somogyi sterilized the contents by heating to boiling over an open flame, then continued the heating for 2 hours in a water bath. Sterilization in this manner is repeated twice more during the next 2 or 3 weeks. A reagent thus prepared keeps indefinitely for practical purposes. The syphon tube is kept filled with the reagent and is closed with a pinchcock. A small rubber bulb filled with alcohol (such as is used for medicine droppers) is slipped on the glass tip of the syphon when not in use. Before the reagent is used, a few drops are allowed to run out from the syphon to rinse away the adhering alcohol.

Somogyi prepares 6 liters of the starch reagent at a time, which suffice for over 1000 diastase determinations. The stock reagent, of course, may also be kept indefinitely if properly sterilized.

Iodine Solution.—(0.002 N aqueous iodine solution containing 2 per cent of KI.)

Dilute 10 c.c. of 0.1 N aqueous iodine solution

to 500 c.c. with a 2 per cent potassium iodide solution.

Technic.—

Into a test tube of 14 to 16 mm. diameter, introduce 4 c.c. of starch solution containing 3 mg. of starch.

Place in constant temperature water bath (40° C.).

After a few minutes, when the reagent has assumed the standard temperature, add 1 c.c. of blood plasma or serum under examination.

Start a stop watch simultaneously.

Expedite the mixing of the plasma and starch by blowing the plasma from a 1 c.c. pipette ("to contain") into the reagent, and rinse the pipette once with the mixture.

While the reaction mixture is incubating, measure 0.5 c.c. portions of the iodine solution into test tubes of 7 mm. inside diameter. These test tubes should be fairly uniform.

After 5 to 6 minutes' incubation, withdraw with a pipette a 0.5 c.c. portion of the reaction mixture, and add to one of the iodine tubes for observation of the reaction. View with transmitted light from a standard source. This consists of a 100 watt frosted electric bulb covered by a cardboard screen with a slit in the front. At subsequent time intervals withdraw further 0.5 c.c. samples from the reaction mixture for color tests with iodine, until the red-brown color of erythrodextrin is seen with barely a perceptible tint of purple. At this point, the end point, register the duration of the reaction on the stop watch.

In practice this technic is exceedingly simple. After a few trials one is able to judge with fair approximation the particular shade of purple which appears when the reaction has progressed approximately halfway. If this stage is attained in about 5 minutes (i.e., at the time when the first sample is withdrawn and tested), the subsequent samples are best withdrawn at 2-minute intervals until the red-brown shade becomes predominant over the purple. From this point on, test samples at 1-minute intervals until the end point is reached. Should the reaction have proceeded beyond halfway in 5 or 6 minutes, test samples every minute thereafter. Again, if after 5 minutes the first color test shows more blue than purple, indicating that the cleavage of the starch has just started, allow a greater interval of time to elapse before the next test is performed. Experience soon teaches the length of time that can be permitted to elapse between two color tests, so that after some practice the analysis can be carried out with only 2 c.c. of starch solution and 0.5 c.c. of plasma. As a matter of fact, these are the quantities Somogyi has been using for several years.

When the diastase content of the blood is abnormally high, as for example in acute pancreatitis, the color given with iodine will be far past the end point at the 5-minute test. In such instances the determination is repeated with diluted plasma. The extent of the dilution is indicated by the shade of color obtained in the first tests with iodine. Dilutions of 1:2 to 1:15 may be necessary. For dilution of the plasma 0.5 per cent sodium chloride solution is used.

Somogyi found that in many pathologic conditions, notably in those resulting in impaired liver function, the blood diastase is lower than in the healthy subject. In such cases the reaction time extends beyond 30 minutes, not infrequently up to 60 minutes and even longer.

Calculations.—

From the equation $D = K \times 1/(t \times v)$, the diastatic activity can be obtained once K , the constant factor, has been experimentally determined. As stated before, under the standard conditions described, $K = 1600$. Thus, for example, when 12 minutes are required for 1 c.c. of plasma to digest 3 mg. of starch to the selected end point of the reaction, $D = 1600/12 = 133$, or, when the reaction time for 1 c.c. of a 1:4 dilution of plasma (actually 0.25 c.c. of plasma) is 13 minutes, $D = 1600/(13 \times 0.25) = 492$. If 1.5 mg. of starch are employed for the assay of very low diastatic activity, $K = 800$, provided that the reaction time does not exceed 30 minutes.

The values obtained in this manner are in close agreement with those obtained by the copper reduction method. There is no need, therefore, to introduce any specific "diastase unit," to express diastatic activity as determined on the basis of the amylolytic activity of the enzyme.

The numerical value of K depends on several factors. The foremost among these is the quality of the substrate. Different samples of the starch reagent, prepared in Somogyi's laboratory from various batches of corn or rice starch in the course of several years, showed no substantial variations in the value of K .

Older samples, however, that stood in the laboratory for several years after being washed, yielded somewhat turbid solutions, and a value of 1800 to 2000 for K. It is noteworthy that potato starch cannot be used in this procedure because it gives, with iodine, indefinite, pale color shades.

Another factor, the subjective element that enters into the appraisal of color shades, may appreciably affect the reading of the end point; i.e., the time factor. The use as the standard source of light of some device that differs from Somogyi's (as for example the use of a blue glass bulb) is still another factor which affects the reading of the end point. All these variables are absorbed in the value of K as its components, and as a consequence they can be ruled out as possible sources of error by determining K under any particular conditions selected as standards. Thus, instead of considering the technic as described above as a stereotyped formula, every competent worker may adapt it to special needs by selecting standard starch concentrations (one may increase it up to 0.4 per cent) and volumes which differ from those just described. The two main limiting factors are the requirements that linear proportionality must obtain between enzyme concentration and reaction time, and that the reaction mixture must be fairly transparent.

This method cannot be used for assay of whole blood, of strongly lipemic or excessively icteric plasma, etc. In such instances one must, according to Somogyi, resort to the copper reduction method.

Gray, Probstein, and Heifetz⁷ reported on the value of low blood diastase determinations as an index of impaired hepatic function. Various workers have reported abnormally high levels of blood diastase in cases of diseases of the biliary tract, acute obstruction of the common bile duct,⁸ disease of the gall bladder⁹ and catarrhal jaundice.¹⁰ In the light of the observations of Gray et al., it is likely that in most of these reported cases acute pancreatic involvement was the actual cause of the increased diastatic activity; in some instances renal insufficiency might have been discovered as the cause had the urine diastase-blood diastase ratio been determined.¹¹ Other workers have found that jaundice per se does not change the level of the blood diastase.¹² Gray and his fellow workers have verified these facts. They found that the diastatic activity of blood in cases of disease of the biliary tract tends toward subnormal levels, probably as a result of involvement of the liver. The occasional finding of an elevated blood diastase level points to probable acute pancreatic involvement, a well-known occurrence in disease of the biliary tract.¹³ The fall in blood diastase level is always accompanied by a decrease in the urinary excretion of the enzyme, although the parallelism between the two changes is not strict.

⁷Gray, S. H., Probstein, J. G., and Heifetz, C. J.: *Arch. Int. Med.* **67**: 805, 1941.

⁸(a) Millbourn, E.: *Acta chir. Scandinav.* **77**: 523, 1936.

(b) Foged, J.: *Am. J. Surg.* **27**: 439, 1935.

(c) Branch, C. D., and Zollinger, R.: *ibid.* **41**: 233, 1938.

⁹(a) Cohn, E.: *Arch. f. Verdauungskr.* **39**: 199, 1926.

(b) Kaczander, P.: *Deutsche med. Wchnschr.* **57**: 1103, 1931.

(c) Millbourn: *loc. cit.* 8a.

¹⁰Cameron, G.: *J. Metab. Research* **5**: 243, 1924. Cohn—9a.

¹¹Heifetz, C. J., Probstein, J. C., and Gray, S. H.: *Arch. Int. Med.* **67**: 819, 1941.

¹²(a) Stocks, P.: *Quart. J. Med.* **9**: 216, 1916.

(b) Elman, R., Arneson, N., and Graham, E. A.: *Arch. Surg.* **19**: 943 (Pt. 1), 1929.

(c) Fricker, E.: *Schweiz. med. Wchnschr.* **56**: 129, 1936.

Millbourn: *loc. cit.* 8a, Foged: *loc. cit.* 8a.

¹³Gray, S. H., Probstein, J. G., and Heifetz, C. J.: *Ann. Surg.* **108**: 1029, 1938.

Normal diastase values, determined by Somogyi technic, range between 80 and 150; values between 60 and 80 and between 150 and 180 are on the borderline of normal. Values below 60 are considered definitely subnormal.

Gray found that in *involvement of the liver and the bile ducts* there is definite and extensive diminution of hepatic function. In cases of diseases of the liver and the bile ducts, including single and multiple hepatic abscesses, acute catarrhal jaundice, obstruction of the common bile duct, malignant conditions of the liver and the bile ducts, cirrhosis of the liver, enlargement of the liver of undetermined origin, and miscellaneous diseases of the liver, there was a tendency in the direction of subnormal diastatic activity. The most notable decreases occurred in cases of abscess of the liver and of a malignant condition.

In a study of *diseases of the gall bladder*, they found that in acute cholecystitis there was a greater lowering of the diastase level than was the case in chronic cholecystitis. Acute infections seem to exert on the liver, therefore, a more severe toxic effect than do chronic infections.

In *infections in general*, there is a systemic response by the liver as a detoxifying agent. As a result of these responses, cloudy swelling, fatty degeneration, and necrosis of the liver may result. It was not surprising, therefore, that in many of the cases of both generalized and localized infections there were subnormal diastase values, according to Gray. It was found that in pneumonia there were low diastase levels.

Diabetes mellitus was studied with respect to diastase values in 736 patients. It was found that the more severe the diabetes, the lower was the diastatic activity of the blood.

Toxemias of pregnancy.—In these cases there was a tendency in the direction of subnormal diastase levels.

Hyperthyroidism.—These workers found that increased thyroid activity is accompanied by a decrease in the diastatic activity of the blood. This was attributed to the involvement of the liver that usually accompanies thyrotoxicosis.

Further clinical studies on blood diastase by Heifetz, Probst, and Gray,¹⁴ using the analytic technic of Somogyi, showed the following with respect to elevation of values:

1. Elevated blood diastase values are found in cases of acute pancreatitis, perforation of peptic ulcer into or near the pancreas, occlusion of the salivary duct, suppuration of the salivary glands, and impairment of renal function.

2. When the blood diastase level is elevated to 1,000 or higher, in general it is safe to assume the presence of acute pancreatitis. Rare exceptions were encountered in cases of occlusion of the salivary duct. The clinical picture in such cases will allow differentiation without difficulty.

3. When moderate degrees of elevated blood diastase, e.g., 200 to 1,000, are found, it becomes indispensable to determine the urine diastase-blood diastase ratio. If this ratio is lowered to unity or below unity, the primary cause is deficient renal excretion of diastase. The increase of the diastase level under

¹⁴Heifetz, C. J., Probst, J. G., and Gray, S. H.: Arch. Int. Med. 67: 819, 1941.

such circumstances seldom exceeds 500, but in a few cases of exceptionally severe renal insufficiency in a group of 111 cases blood diastase levels as high as 1,000 were observed.

4. Moderate increases in blood diastase also may occur in cases of perforation of posterior peptic ulcer into or near the pancreas.

5. Gray and his fellow workers advise that diastase determinations be judiciously correlated with clinical observations and other laboratory data in order to offer an important aid to diagnosis.

As previously reported by Elman,¹⁵ in pancreatic injury, such as acute pancreatitis, obstruction of pancreatic ducts, etc., there is an increase in both blood and urinary amylase. According to Cole and Elman,¹⁶ the blood amylase has been studied with a view of determination of changes in pancreatic secretion.

According to Cole and Elman, numerous studies by various observers indicate that determinations of blood amylase are valuable in the diagnosis of acute pancreatic disease; for instance, in pancreatic cyst, in acute pancreatic necrosis, and in acute pancreatitis without necrosis the blood amylase has been found to be elevated to many times the normal value. In the case of acute pancreatic disease the level of blood amylase depends to a great extent on the stage of the pancreatic disease. When transient inflammation or obstruction to the duct occurs, the blood amylase will be high only at the height of the attack and subsides rapidly with the resolution of the acute process. Thus, they state, a determination of blood amylase at a time when the patient is suffering no acute symptoms will probably reveal a normal value and give no indication of the true nature of the disease. This is important because in many instances the attack may be of only a few hours' duration. In such cases the blood amylase to be of value might be classed as an emergency procedure. On the other hand, these authors emphasize that as a negative test the measurement of blood amylase at the time of acute symptoms may also be of value, particularly when acute pancreatic necrosis is suspected, a condition which demands operative treatment. This is very important if the acute upper abdominal pain is actually due to coronary disease. In such a case, a normal blood amylase will rule out the possibility of pancreatic disease; this will obviously greatly simplify the problem of therapy and thus avoid the likelihood of an ill-advised operation which, of course, is likely to be fatal.

The distribution of diastase (amylase) in serum, plasma, whole blood and red blood cells has been studied by Bernard and Rosen.¹⁷ They used Somogyi's methods¹⁸ just described with the exception that the Benedict¹⁹ 1931 copper method for the estimation of sugar in the tungstic acid filtrates was used. Their determinations were made on 0.5 c.c. of serum, plasma, whole blood, and red cells. They found that the diastase values of whole blood are too low to be of any diagnostic value; further that the red cells contain no diastase, the values obtained being between 0 and 13, and are quite independent of the red cell

¹⁵Elman, R.: *Graham's Surgical Diagnosis*, Vol. II, Philadelphia, 1930, W. B. Saunders Co., p. 550.

¹⁶Cole and Elman: *Textbook of General Surgery*, New York, 1936, D. Appleton-Century Company, p. 600.

¹⁷Bernhard, A., and Rosen, M. J.: *Proc. Soc. Exper. Biol. and Med.* 48: 575, 1941.

¹⁸Somogyi, M.: *J. Biol. Chem.* 125: 399, 1938.

¹⁹Benedict, S. R.: *J. Biol. Chem.* 92: 141, 1931.

volume; also that the red cells contain no amylase has been reported by Morse¹ in experiments on dogs. The final conclusion of these workers was that the plasma values approach those of the serum, while the whole blood values are considerably lower. The red cells contain no diastase.

SERUM LIPASE

The determination of serum lipase is of value in the diagnosis of diseases of the pancreas. The Loevenhart method, as suggested by Cherry and Crandall,² has been modified by Johnson and Bockus.³ This method depends on the estimation of fatty acid produced by the hydrolysis of an olive oil emulsion on which the lipase in the blood serum has been acting during a twenty-four-hour period under optimum conditions of pH and temperature.

Reagents.—

Buffer Solution.—

Mix 10 c.c. of M/3 solution of disodium hydrogen phosphate (Na_2HPO_4) (see page 23, Molar Solutions).

and 3 c.c. of a M/3 solution of potassium dihydrogen phosphate (KH_2PO_4) (see page 23, Molar Solutions).

50% Olive Oil Emulsion.—

Prepare a 50% emulsion of olive oil using 5% solution of acacia as the emulsifying agent, and adding 0.2% of sodium benzoate as a preservative. This solution may be obtained from the Abbott Laboratories.

95% Ethyl Alcohol.—

1% Alcoholic Solution of Phenolphthalein.—(See page 1199.)

N/20 Sodium Hydroxide Solution.—

Dilute 50 c.c. of N/1 sodium hydroxide solution (page 25) to exactly 1,000 c.c. with distilled water in a liter volumetric flask.

Technic.—

Place 3 c.c. of distilled water in each of two test tubes labeled, respectively, "A," and "B."

Add 1 c.c. of serum to each tube and shake.

Place tube A in a water bath at 100° C. for five minutes to inactivate the lipase. This is the control tube. Be sure to cool tube A before proceeding to the next step.

Add 0.5 c.c. of buffer solution to each tube.

Add 2 c.c. of 50% olive oil emulsion to each tube and shake.

Incubate at 37.5° C. for 24 hours.

Add 3 c.c. of 95% alcohol and 2 drops of 1% solution of phenolphthalein to each tube.

Titrate each tube to a faint pink, using N/20 sodium hydroxide solution.

Subtract the value for control tube A from that for specimen tube B.

Express the results in terms of c.c. of N/20 sodium hydroxide.

Comfort⁴ stated that significant values are in excess of 1.5 c.c. of N/20 sodium hydroxide solution. Johnson and Bockus' figure showed that some importance might be attached to any figure above 1.0 c.c. Their highest value, 5.5 c.c., is somewhat lower than the highest value reported by Comfort. They were unable to account for their inability to obtain, by titration, values in excess of approximately 5.0 c.c. in cases of acute pancreatitis. Baxter, Baxter, and

¹Morse, W.: *Proc. Am. J. Biol. Chem.* 55: 27, 1923.

²Cherry, I. S., and Crandall, L. A.: *Am. J. Physiol.* 100: 266, April, 1932.

³Johnson, T. A., and Bockus, H. L.: *Arch Int. Med.* 66: 62-77, July, 1940.

⁴Comfort, M.: *Proc. Staff Meet., Mayo Clin.* 10: 810, Dec. 18, 1935.

McIntosh¹ reported values ranging from 3.0 to 5.5 c.c. in experimentally induced acute pancreatitis in dogs. These values were not dissimilar to Johnson and Bockus' maximum figures for acute pancreatitis. In their analysis of values for serum lipase in 371 cases, Johnson and Bockus found the following:

In *acute pancreatitis*, of eleven cases, nine had high values at some time during the course of the illness and normal values were obtained in two cases. It now seems certain that ample clinical and experimental evidence exists to demonstrate that the amount of lipase in the serum is frequently increased in acute pancreatitis. This is due to two possible mechanisms: (1) absorption of lipase directly into the blood stream from necrotic pancreatic tissue; and (2) absorption of lipase from the smaller pancreatic ducts which have been blocked by edema secondary to adjacent inflammation. In *carcinoma of the pancreas* values above 1 c.c. were obtained in 62.7 per cent. It is the belief of Johnson and Bockus that the test for lipase will be of distinct value in *obscure cases of jaundice*. An elevated value for lipase possibly can be looked on as militating against a diagnosis of so-called *catarrhal jaundice*. In hyperbilirubinemia there does not seem to be any relation to the level of serum lipase. In *chronic hepatic disease*, Comfort² reported increased values for serum lipase in 13 per cent of his cases. He believes that the increase resulted either from destruction of the hepatic parenchyma or from associated pancreatitis. Johnson and Bockus found 29 per cent of 24 cases of *cirrhosis of the liver* were associated with elevated lipase values. They found an elevation of serum lipase in *intestinal obstruction* but could not explain it.

ACID AND ALKALINE PHOSPHATASE

Modified Method of Bodansky³

I. Alkaline Phosphatase.—

Reagents.—

10% Trichloroacetic Acid Solution.—Dissolve 10 gm. trichloroacetic acid, reagent grade, in distilled water in a 100 c.c. volumetric flask, and dilute to 100 c.c. with distilled water.

Stock Phosphate Solution.—This is the same as the Standard Phosphate Stock Solution as on page 451.

Standard Phosphate Solution.—(1 c.c. = 0.01 mg. P.) Dilute 10 c.c. of Stock Phosphate Solution to 100 c.c. with distilled water in a 100 c.c. volumetric flask. Add a few drops of chloroform.

7.5% Sodium Molybdate Solution.—Dissolve 7.5 gm. phosphorus-free sodium molybdate in distilled water and dilute to 100 c.c. with distilled water.

10 N Sulphuric Acid Solution.—See page 24.

Molybdic Sulphuric Acid Reagent.—

Solution A.—

To 50 c.c. of 7.5% sodium molybdate solution
add 50 c.c. of 10 N sulphuric acid and mix well.

Stock Stannous Chloride Solution.—See page 451.

¹Baxter, H., Baxter, S. G., and McIntosh, J. F.: *Am. J. Digest. & Nutrition* 5: 423, Sept., 1938.

²Comfort, M.: *Proc. Staff Meet., Mayo Clin.* 10: 810, Dec. 18, 1935.

³*Am. J. Clin. Path., Tech. Supp.* 1: 51, 1937.

Am. J. Clin. Invest. 17: 473, 1938.

Canad. M. A. J. 31: 376, 1934.

Dilute Stannous Chloride Solution.—Dilute 1 c.c. of the stock solution to 200 c.c. with distilled water and keep in a brown bottle. This solution must be used within 5 days after making. Better results are obtained with this fresh reagent than with solutions which have been held more than 5 days.

Sodium Glycerophosphate Substrate, pH 8.6.—

Place in correct order in a 100 c.c. volumetric flask
about 3 c.c. petroleum ether (b.pt. 20 to 40° C.)
about 80 c.c. distilled water
0.5 gm. sodium beta-glycerophosphate
and 0.424 gm. sodium diethylbarbiturate, U.S.P., and

dilute to 100 c.c. with distilled water. Read at the interface between the substrate and the petroleum ether. Mix and adjust the reaction to pH 8.6, using either N/10 hydrochloric acid or N/10 sodium hydroxide as indicated. Empty out of doors into a 100 c.c. glass-stoppered Pyrex bottle containing about 1 inch of petroleum ether. Keep in the refrigerator in quantities not to exceed 100 c.c. per bottle.

a. Preparation of the Serum.—

Collect about 5 c.c. of whole blood, allow to clot at room temperature, centrifuge, remove serum, recentrifuge the serum, and decant. The serum may be kept in the refrigerator for several hours if it is impossible to use it at once. After 24 to 48 hours in the refrigerator serum phosphatase is about 10 to 15 per cent higher. If serum is kept at room temperature, the results are subject to an error of plus or minus 20 per cent. One drop of toluene may be added to the serum as a preservative.

b. Preparation of Filtrate for Serum Inorganic Phosphorus.—

Pipette into a large test tube 1 c.c. of serum.
Add 4 c.c. of 10% trichloroacetic acid.
Mix and set aside for a few minutes.
Filter through 9 cm. Whatman No. 44 filter paper or other "ashless" grade.

c. Preparation of Filtrate for Total Inorganic Phosphorus (Serum Inorganic Phosphorus and the Inorganic Phosphorus Liberated From the Substrate).—

Place 10 c.c. of substrate in a glass-stoppered or rubber-stoppered test tube.
Place in a water bath at 37° C. until warm.
Add 1 c.c. serum, keeping the tip of the pipette about 1 cm. above the surface of the liquid. Invert once, mixing the contents thoroughly, but without undue aeration.
Replace in the water bath.
At the end of exactly one hour, remove tube from the water bath and cool in ice water for about 2 minutes.
Add 9 c.c. of 10% trichloroacetic acid, mix well, and allow to stand for a few minutes. Filter.
If the serum contains a high phosphatase value, dilute the final mixture with an additional amount of 10% trichloroacetic acid.

Technic.—

Determination of Inorganic Phosphorus.—

Place 2 c.c. of filtrate (for inorganic phosphorus, above) in a 50 c.c. Erlenmeyer flask labeled "U" for unknown.

Place 2 c.c. standard phosphate solution (containing 0.02 mg. P) in a similar flask labeled "S" for standard.

Add to each flask 5 c.c. distilled water
and 2 c.c. molybdic sulphuric acid reagent, solution A,

Mix. *Immediately, blow in*, from a pipette, 1 c.c. of dilute stannous chloride solution. Mix at once and allow to stand at room temperature for 15 minutes. Read in the colorimeter.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 5 = \text{mg. inorganic P per 100 c.c. serum.}$$

Determination of Total Inorganic Phosphorus (Serum Inorganic Phosphorus and the Inorganic Phosphorus Liberated From the Substrate).—

Carry out the same procedure as for the determination of inorganic phosphorus above, but use 4 c.c. of the filtrate made with the substrate and add only 3 c.c. distilled water.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 10 = \text{mg. total inorganic phosphorus per 100 c.c. serum.}$$

Calculation for Alkaline Phosphatase.—

Total inorganic phosphorus - serum inorganic phosphorus = units of alkaline phosphatase activity per 100 c.c. of serum (if the hydrolysis was continued for 1 hour). (mg. of inorganic phosphorus liberated from glycerophosphate at pH 8.6.)

II. Acid Phosphatase.—

Reagents.—

The reagents for this procedure are the same as for alkaline phosphatase with the exception of the sodium glycerophosphate substrate.

Sodium Glycerophosphate Substrate pH 6.4.—

Place in correct order in a 100 c.c. volumetric flask
about 3 c.c. petroleum ether (b.pt. 20 to 40° C.)
about 80 c.c. distilled water

and 0.5 gm. sodium beta-glycerophosphate, and dilute to 100 c.c. with distilled water.

Read at the interface between the substrate and the petroleum ether. Mix and adjust the reaction to pH 6.4 using N/10 hydrochloric acid or N/10 sodium hydroxide as indicated. Empty out of doors into a 100 c.c. glass-stoppered Pyrex bottle containing about 1 inch of petroleum ether. Store in the refrigerator in quantities not to exceed 100 c.c. per bottle.

Technic.—

Follow the method given for alkaline phosphatase above, substituting the pH 6.4 substrate for the substrate of pH 8.6. Use 2 c.c. of the filtrate for inorganic phosphorus (p. 386) and 7 c.c. of the filtrate for total inorganic phosphorus, using no water in the determination of total inorganic phosphorus.

Calculation for Acid Phosphatase.—

Inorganic Phosphorus.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 5 = \text{mg. of inorganic phosphorus per 100 c.c. serum.}$$

Total Inorganic Phosphorus.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 5.7 = \text{mg. total phosphorus per 100 c.c. serum.}$$

Acid Phosphatase.—

Total inorganic phosphorus - serum inorganic phosphorus = units of acid phosphatase activity per 100 c.c. serum (if hydrolysis was continued for 1 hour). (mg. inorganic phosphorus liberated from glycerophosphate at pH 6.4.)

Table 25 presents the same information in a more concise form.

TABLE 25.—ACID-ALKALINE PHOSPHATASE (BODANSKY)

	TOTAL INORGANIC PHOSPHORUS FOR ALKALINE PHOSPHATASE	TOTAL INORGANIC PHOSPHORUS FOR ACID PHOSPHATASE	SERUM PHOSPHORUS	STANDARD
Substrate—pH 8.6	10 c.c.	—	—	
Substrate—pH 6.4	—	10 c.c.	—	
	Water bath at 37° C. until warm			
Serum	1 c.c.	1 c.c.	1 c.c.	
	Water bath 37° C. for 1 hour. Cool in ice water			
10% Trichloroacetic acid	9 c.c.	9 c.c.	4 c.c.	
	Filter	Filter	Filter	
Filtrate	4 c.c.	7 c.c.	2 c.c.	—
Standard solution	—	—	—	2 c.c.
Distilled water	3 c.c.	—	5 c.c.	5 c.c.
Molybdic sulphuric reagent	2 c.c.	2 c.c.	2 c.c.	2 c.c.
Dilute stannous chloride	1 c.c.	1 c.c.	1 c.c.	1 c.c.
	Allow to stand 15 min.—Read			
	$\frac{RS}{RU} \times 10 = \text{mg.}$	$\frac{RS}{RU} \times 5.7 = \text{mg.}$	$\frac{RS}{RU} \times 5 = \text{mg.}$	

Calculation.—

Total Inorganic P for } - Serum Phosphorus = Bodansky units of alkaline phosphatase per 100 c.c. serum.

Normal = 1.4563 to 3.884.

Total Inorganic P for } - Serum Phosphorus = Bodansky units of acid phosphatase per 100 c.c. serum.

Normal = 0.0437 to 0.116.

Normal Total Units.—1.5 to 4 Bodansky units adults, average 2.7
5 to 12 or 4 to 14 children, average 8.

Serum Phosphatase Determination, Roe and Whitmore¹

Reagents.—

Trichloroacetic Acid.—

A pure grade of trichloroacetic acid, free from phosphate, must be used. Prepare accurately a 5 per cent and a 10 per cent solution.

Buffered Glycerophosphate Substrate.—

Dissolve 2.15 gm. of sodium glycerophosphate and 2.12 gm. of sodium diethyl barbiturate in 500 c.c. of distilled water. Cover with a 3 cm. layer of petroleum ether and keep in a refrigerator.

Molybdate Reagent.—

Dissolve 2.5 gm. of ammonium molybdate, c.p., in 100 c.c. of distilled water.

Aminonaphtholsulphonic Acid Reagent.—

Dissolve 30 gm. of sodium bisulphite, c.p., and 1 gm. of sodium sulphite, c.p., in 200 c.c. of distilled water. Add 0.5 gm. of purified 1-amino-2-naphthol-4-sulphonic acid, and mix thoroughly. Filter. Place in a dark bottle. This reagent should be freshly prepared about once a month.

¹Roe, J. H., and Whitmore, E. R.: Am. J. Clin. Path. 8: 233, 1938.

Standard Phosphate Solutions.—

- A. Stock solution: Dissolve 4.388 gm. of pure dry monobasic potassium phosphate (KH_2PO_4) in 1 liter of distilled water. Add 5 c.c. of chloroform to prevent mold formation. One cubic centimeter of this solution contains 1 mg. of phosphorus.
- B. Dilute standards for blood phosphatase: Dilute 1 c.c., 2 c.c., 3 c.c., and 4 c.c. of the stock solution to 500 c.c. with the 5 per cent trichloroacetic acid prepared above. Five cubic centimeters of these diluted standards contain 0.01, 0.02, 0.03, and 0.04 mg. of phosphorus, respectively.

Technic.—

Pipette 10 c.c. of the glycerophosphate substrate into a test tube and place in a beaker of water at 37° C. for a few minutes. Add 1 c.c. of serum (or plasma), mix thoroughly, replace in the beaker of water, and set the beaker in an incubator at 37° C. for 1 hour. After 1 hour remove from the incubator and add 9 c.c. of 10% trichloroacetic acid. Mix thoroughly, let stand for 2 minutes, and filter through a phosphate-free filter paper (Whatman's No. 44). This filtrate is used for determining the original phosphate of the serum plus the phosphate resulting from hydrolysis of glycerophosphate by the phosphatase. Label it phosphatase filtrate.

To 1 c.c. of serum add 9 c.c. of 5 per cent trichloroacetic acid, mix thoroughly, let stand for 2 minutes, and filter through a phosphate-free filter paper. This filtrate is used to determine the inorganic phosphate of the serum. Label it control filtrate.

For the determination select tubes graduated with a 10 c.c. mark. Place 5 c.c. of the control filtrate in a tube labeled appropriately and 5 c.c. of the phosphatase filtrate in another tube.

Prepare standards by placing in tubes 5 c.c. portions of the diluted standard phosphate solutions, making standards available which contain 0.01, 0.02, 0.03, and 0.04 mg. of phosphorus per 5 c.c.

To each of the tubes containing standard or filtrate add 1 c.c. of molybdate reagent and 1 c.c. of aminonaphtholsulphonic acid reagent. Make all tubes up to 10 c.c. with distilled water and mix thoroughly. After 5 minutes read in a colorimeter, selecting for each unknown the standard which most closely matches it.

Calculation.—

For control filtrate:

$$\frac{S}{U} \times S_1 \times 200 = \text{mg. per 100 c.c. of serum.}$$

For phosphatase filtrate:

$$\frac{S}{U} \times S_1 \times 400 = \text{mg. of P per 100 c.c. of serum plus P liberated by the enzyme.}$$

S = reading of standard; U = reading of unknown; S_1 = mg. P in standard selected.

To obtain the units of phosphatase, subtract the value of the control filtrate from that of the phosphatase filtrate.

Serum Phosphatase, Spectrophotometric Method,* Sigma

The Sigma Chemical Company¹ has improved p-nitrophenyl phosphate. They offer a white crystalline p-nitrophenyl phosphate which is stable for some time when it is kept cold. It is supplied in capsules containing 100 mg. each. One capsule is sufficient for 48 determinations or 24 if not made in a group.

*Jeremiah, B.: Lab. Digest 15: 11, 1952.

¹Sigma Chemical Company, 4648 Easton Ave., St. Louis 13, Mo.

It is not necessary to prepare a filtrate nor wait for color development, since the color develops immediately upon the addition of the reagent which stops the phosphatase activity.

Any spectrophotometer or any colorimeter which is fitted with a light filter that transmits light with a sharp peak in the range of 400 to 420 mμ can be used.

Principle.—

Para-nitrophenyl is colorless in solution. Hydrolysis of the phosphate group liberates p-nitrophenol which is highly colored in alkaline solution. The rate of hydrolysis of p-nitrophenyl phosphate is proportional to the concentration of the enzyme for at least 30 minutes.

Hemoglobin, when present in serum as a result of hemolysis, absorbs less light at 410 than at 400 mμ which is the reason for preferring 410 mμ even though peak absorption of p-nitrophenyl is at 400 mμ. Hemoglobin does not absorb quite the same amount of light in acid as in alkaline solution, so excessive hemolysis should be avoided. For acid phosphatase determinations, any visible hemolysis must be avoided because of the high content of this enzyme normally present in the red cells.

Acid or alkaline phosphatase tests can be completed in 45 minutes by this method. The actual working time is 15 minutes or less.

Preparation of the Calibration Curve.—

Use p-nitrophenol produced by Sigma Chemical Co.
Prepare test tubes as in Table 26.

TABLE 26

TUBE NO.	WORKING STANDARD NO. 104-2 C.C. 0.05 mM*/L. P-NITROPHENOL	DISTILLED WATER C.C.	0.2N NaOH C.C.
1	1	9	1.1
2	2	8	1.1
3	4	6	1.1
4	6	4	1.1
5	8	2	1.1
6	9	1	1.1
7	10	0	1.1
Mix			

*Millimoles

Adjust the spectrophotometer to 410 mμ wave length.

Set the spectrophotometer to 100% transmission with distilled water in a cuvette 13 by 13.

Read the percentage of transmission of each of the above solutions. Pour the solutions into 13 by 13 cuvette before reading.

Plot the transmission readings on semi-log paper.

Transfer the readings to a calibration chart.

Multiply the alkaline phosphatase by 0.234 mM to obtain the acid phosphatase.

Reagents.—

0.4% p-Nitrophenyl Phosphate, Stock Solution.—

Dissolve the contents of 1 capsule (100 mg. p-nitrophenyl phosphate) in 25 c.c. of distilled water.

For Alkaline Phosphatase.—

Buffered Substrate Solution.—

Dilute 1 volume of 0.4% stock substrate solution with 1 volume of Sigma buffer No. 104-5.

For Acid Phosphatase.—

Buffered Substrate Solution.—

Dilute 1 volume of 0.4% stock substrate solution with 1 volume of Sigma buffer No. 104-4.

Store completed substrates in a refrigerator. Freeze to maintain longer stability. **Technic.—**

Carry out the technic for acid and alkaline phosphatase as given in Table 27.

TABLE 27.—TECHNIC OF SIGMA PHOSPHATASE—ACID AND ALKALINE

	ACID PHOSPHATASE			ALKALINE PHOSPHATASE	
	UNKNOWN	REAGENT BLANK	SERUM BLANK	UNKNOWN	REAGENT BLANK
Buffered substrate (acid)	1 c.c.	1 c.c.	0.0	0.0	0.0
Buffered substrate (alkaline)	0.0	0.0	0.0	1 c.c.	1 c.c.
Place in a water bath at 37° C. for 5 minutes.					
Serum	0.2 c.c.	0.0	0.2 c.c.	0.1 c.c.	0.0
Distilled water	0.0	0.2 c.c.	0.0	0.0	0.1 c.c.
Place in a water bath at 37° C. for 30 minutes. Remove and add promptly					
N/10 NaOH	4.0 c.c.	4.2 c.c.	5.0 c.c.	0.0	0.0
N/50 NaOH	0.0	0.0	0.0	10.0 c.c.	10.0 c.c.
Read each sample in 13 × 13 cuvette at 410 mμ wave length					
	Read unknown against reagent blank		Read serum against water	Read unknown against reagent blank, then add	
Concentrated HCl	0.0	0.0	0.0	2 drops	2 drops
Read again as above					

Calculations.—**Acid Phosphatase:**

Acid phosphatase of serum equals reading of the unknown minus the reading of the serum blank.

Determine the acid phosphatase values from the calibration chart.

Alkaline Phosphatase:

Alkaline phosphatase of serum equals alkaline reading minus (alkaline plus HCl).

Determine the alkaline phosphatase values from the chart for both readings.

One millimolar (mM) unit of phosphatase activity liberates 1 millimole of p-nitro-phenyl per liter of serum per hour.

Millimolar units × 1.8 = Bodansky units (approximately).†

Normal adult sera range from 0.8 to 2.3 mM units, total.

Normal children's sera range from 2.8 to 6.7 mM units, total.

Serum Phosphatase Determination**King and Armstrong^{1*}**

Reagents.—(Note: All chemicals must be of analytical reagent quality.)

pH 5.0 Buffer Substrate.—

Dissolve 1.09 gm. disodium-monophenyl-phosphate (Paul Lewis, Inc.) in 300 c.c. of 0.2 N acetic acid in a liter volumetric flask.

Dilute to 1000 c.c. with 0.2 N sodium acetate.

This constitutes a 0.005 M solution of disodium-monophenyl-phosphate.

Add 5 c.c. chloroform. Keep on ice.

*This is a modification of the King and Armstrong method and is published through the courtesy of the Paul Lewis Laboratories, 918 North Fourth St., Milwaukee, Wisconsin. A publication by this company was prepared through the courtesy of C. V. Hodges, M.D., Department of Surgery, University of Chicago. Further information may be obtained by addressing the Paul Lewis Laboratories.

†Normal values are given on page 388.

¹King, E. J., and Armstrong, A. R.: Canad. M. A. J. 31: 376, 1934.

0.2 N Sodium Acetate.—

Dissolve 27.22 gm. sodium acetate ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$) in enough distilled water in a liter volumetric flask to make 1000 c.c. solution.

0.2 N Acetic Acid.—

Add 11.3 c.c. glacial acetic acid to enough distilled water in a liter volumetric flask to make 1000 c.c. solution.

pH 9.3 Buffer Substrate.—

Dissolve 1.09 gm. disodium-monophenyl-phosphate (Paul Lewis, Inc.) and 10.3 gm. sodium barbital (Paul Lewis) in distilled water in a liter volumetric flask and dilute to 1000 c.c. with distilled water.

Add 5 c.c. chloroform. Keep on ice.

Phenol Reagent of Folin and Ciocalteu.²—

See page 305 for method of making. The reagent should have no greenish tint. It should be kept well protected from dust, because organic materials will gradually produce slight reductions. Dilute this reagent one in three (250 c.c. reagent and 750 c.c. distilled water.)

20% Sodium Carbonate Solution.—

Dissolve 200 gm. anhydrous sodium carbonate (Na_2CO_3) in distilled water and dilute to 1000 c.c. with distilled water.

Stock Phenol Solution.—

Place 1 gm. crystalline phenol in a liter volumetric flask.

Dissolve in 0.1 N hydrochloric acid and dilute to 1000 c.c. with 0.1 N hydrochloric acid.

Transfer 25 c.c. of this solution to a 250 c.c. volumetric flask, add 50 c.c. of 0.1 N sodium hydroxide, and heat to 65° C.

To the hot solution, add 25 c.c. of 0.1 N iodine solution.

Stopper the flask and allow it to stand at room temperature for from 30 to 40 minutes.

Add 5 c.c. of concentrated hydrochloric acid.

Titrate the excess of iodine with 0.1 N sodium thiosulphate solution.

Dilute Stock Standard Phenol Solution.—

Each c.c. of 0.1 N iodine (c.c. iodine added minus c.c. of thiosulphate used in titration) corresponds to 1.567 mg. of phenol. On the basis of the titration, dilute the phenol solution so that 1.0 c.c. contains 0.1 mg. of phenol.

This dilute stock standard phenol solution (10 mg. per 100 c.c. solution) is used in the preparation of the standard curve (see below).

This solution will keep for at least three months in the refrigerator.

Standardization of Iodine and Sodium Thiosulphate.—(See page 27.)**Preparation of Standard Curve.—**

Prepare a series of solutions, diluting the dilute stock standard phenol solution (10 mg. in 100 c.c.) with distilled water in appropriate amounts so that 5.0 c.c. portions will contain successive amounts of phenol ranging from 0.005 mg. to 0.1 mg. (0.05 c.c. of dilute standard contains 0.005 mg. phenol).

Take 5 c.c. portions of each dilution in separate 25 c.c. volumetric flasks.

Add 4.5 c.c. diluted phenol reagent to each.

Add 2.5 c.c. of 20% sodium carbonate to each. (Note the time when the sodium carbonate is added to the first flask. Observe a convenient time interval, 30 seconds to 1 minute, between additions to subsequent flasks, so that the same time sequence may obtain in taking colorimeter readings.

Dilute to 25 c.c. with distilled water.

Pour 10 to 15 c.c. from each volumetric flask into separate colorimeter tubes.

Read in exactly 20 minutes from the time the sodium carbonate was added to each solution.

²Folin, O., and Ciocalteu, V.: J. Biol. Chem. 73: 627, 1927.

Take readings on an Evelyn photoelectric colorimeter, using a 6600 Angstrom unit filter and a dim lamp. From these readings, construct a standard curve on semi-logarithmic graph paper. Ordinates are mg. of phenol per 25 c.c. volumetric flask; abscissae are readings on the colorimeter scale. The standard curve should be checked every few months.

Technic.—

Run all tests in duplicate. Bring the buffer-substrate solutions to room temperature before beginning test.

Use 4 test tubes. In two of them, place 10 c.c. each of pH 5.0, and in two, place 10 c.c. each of pH 9.3 buffer-substrate solution.

Allow tubes to remain in a water bath at 37.5° C. until solutions have reached this temperature, at least 5 minutes.

Add 0.5 c.c. serum to each.

Stopper with rubber stopper, invert twice.

Keep in water bath for exactly 30 minutes.

Add 4.5 c.c. of dilute Folin-Ciocalteu reagent.

Invert several times.

Centrifuge for 2 to 3 minutes.

It is important that the Folin-Ciocalteu reagent be added to each tube *exactly 30 minutes* after the serum was added to it, so that a precise period of incubation is observed for each sample.

Control.—

Place in a test tube 10 c.c. of pH 5.0 buffer-substrate solution.

Place in another test tube 10 c.c. of pH 9.3 buffer-substrate solution.

Add to each 4.5 c.c. dilute Folin-Ciocalteu reagent and 0.5 c.c. serum, invert twice, and centrifuge. (It is important that the Folin-Ciocalteu reagent be added *before* the serum.)

Test.—

Into a 25 c.c. volumetric flask, pipette 2 c.c. filtrate from test solution.

Pipette 2 c.c. filtrate from control solution into another 25 c.c. flask.

Add to each 2.5 c.c. of 20% sodium carbonate solution. Carry out at convenient time intervals, 30 seconds to 1 minute, between additions to successive flasks, so that colorimeter readings may be made in the same manner.

Dilute with distilled water to 25 c.c.

Stopper, invert several times, and pour 10 to 15 c.c. of each solution into colorimeter tubes.

Read each solution in the colorimeter *exactly 20 minutes* after the sodium carbonate was added.

Calculation.—

The equivalent mg. of phenol per 25 c.c. of solution for each colorimeter reading is obtained from the standard curve. Then, $\text{mg. of phenol per 25 c.c. of solution} \times 15/2 \times 100/0.5$ (which cancels out to read: $\text{mg. of phenol per 25 c.c. of solution} \times 1500$) equals mg. of phenol per 100 c.c. serum in test or control. The King and Armstrong unit is defined thus: one unit of phosphatase activity is that amount of enzyme which, when allowed to act upon excess disodium-monophenyl-phosphate at proper pH for 30 minutes at 37.5° C., will liberate 1 mg. of phenol. Therefore, $\text{mg. of phenol per 100 c.c. of test serum} - \text{mg. of phenol per 100 c.c. of control (unincubated) serum}$ equals units of phosphatase per 100 c.c. of serum.

(Many laboratories use the older Bodansky method* for determining serum alkaline phosphatase. Sodium-beta-glycero-phosphate is used as a substrate and monosodium-diethyl-barbiturate to buffer to proper pH. One c.c. of serum to be tested is incubated in the buffer-substrate solution at 37° C. for exactly one hour. The enzyme activity is halted by adding 5% trichloroacetic acid. The filtrate is then analyzed for inorganic phosphorus. One unit of alkaline phosphatase activity is defined as that amount of enzyme which, when

*Bodansky, A.: J. Biol. Chem. 101: 93, 1933.

allowed to act upon a given excess of sodium-betaglycero-phosphate at proper pH (8.6) for one hour at 37° C., will liberate 1.0 mg. of phosphorus. Units are expressed per 100 c.c. of serum. The alkaline phosphatase activity of the serum of normal adults ranges from 1 to 4 Bodansky units per 100 c.c. Unpublished data indicate that, allowing for differences in hydrolysis of substrates and in time of incubation, 1 Bodansky unit is approximately equal to 1.8 King and Armstrong units.)

Interpretation.—The normal adult phosphatase activity ranges from 1.5 to 4.0 units per 100 c.c. with an average of 2.7; in children, from 5 to 12 units, with an average of 8. In Paget's disease with multiple bone involvement, the phosphatase activity of the blood serum may be twenty times the normal figure. Smith¹ reported that the blood phosphatase is increased in active rickets, the degree corresponding to the severity of the disease. In obstructive jaundice Roberts² found the blood phosphatase very high, but in all other varieties of jaundice the figure was normal or only slightly raised, even though the patient was deeply jaundiced. Roberts believed that the phosphatase reaction could be used to differentiate obstructive from toxic jaundice. In other conditions of multiple bone pathology the phosphatase test may be of value.

Yaguda³ found in three cases of osteogenesis imperfecta that blood phosphatase activity was normal. In a case of erythroblastic anemia with marked changes in the entire system and in two cases of myeloma, the phosphatase activity was within normal limits. In a series of fractures, the values in Bodansky units ranged from 5.5 to 6.5, average of 6.0, which is above the normal limits for adults. He found that the high figures in fractures with multiple lesions indicated some relationship to the severity of the injury. He did not find any increase in blood phosphatase in arthritis. In general, Yaguda believed that the phosphatase activity of the blood is of clinical significance in pathologic changes in tissues, especially in bone, and that its determination is of definite value in the diagnosis of Paget's disease, active rickets, and jaundice, and is of material aid in following treatment of these conditions.

The report of Roe and Whitmore showed that phosphatase is associated with osteoblastic activity. Hence the determination is of special value in lesions of bone. There have been a number of cases reported indicating where phosphatase determinations are of value in differential diagnosis. Roe and Whitmore showed that phosphatase is increased in osteoblastic metastasis of carcinoma to bone, as in most prostatic cancers. It was not increased in osteolytic metastasis of carcinoma to bone, as in breast cancers. They showed that phosphatase was high in some metastases of mammary cancer to bone, as illustrated in one case. They also showed that there is no increase in phosphatase in carcinoma with metastases other than to bone. Further, they demonstrated that phosphatase studies are of great value in the study of the diseases of the lymphoid and myeloid systems. Since phosphatase is normal or slightly elevated in multiple myeloma, phosphatase determination is of value in differentiating multiple myeloma from hyperparathyroidism. Phosphatase is normal in Ewing's sarcoma; normal or slightly elevated in benign giant cell tumor. Furthermore, Roe and Whitmore

¹Smith, J.: Arch. Dis. Childhood 8: 215, 1923.

²Roberts, W. M.: Brit. M. J. 1: 734, 1923.

³Yaguda, A.: Am. J. Clin. Path. 6: 57, 1936.

reported a case of aleukemic leukemia, lymphocytic type, with extensive involvement of the bones, in which serum phosphatase was in the lower part of the normal range for persons of the age of the patient. They definitely stated: "The serum phosphatase determination here aided greatly in differentiating the case from hyperparathyroidism."

It has been noted by Huggins and others¹ that in carcinoma of the prostate there is a marked increase in the acid phosphatase. It may be as high as 50 per cent or more of the alkaline phosphatase. Normally, the acid phosphatase is about 3 per cent of the alkaline phosphatase. There is a definite increase in the amount of acid phosphatase in carcinoma of the prostate, if no appropriate treatment has been instituted. Note, too, that the estimation of acid phosphatase is important in indicating the effectiveness of the use of hormone therapy or the surgical expedient of castration in the treatment of malignancies of the prostate gland.

Referring further to the work of Huggins,² he has shown that in about 50 per cent of men with prostatic carcinoma, the serum acid phosphatase activity is increased and these increased values are found only in the presence of metastasis. Metastasis may be present without an increase of the phosphatase, but when serum phosphatase is increased, metastasis of the carcinoma is present. In other words, there are false negatives but no false positives in this test.

BODY WATER AND ELECTROLYTES IN HEALTH AND DISEASE*

Morbidity and mortality from disease and injury in both medical and surgical diseases have been reduced in recent years because of the application of a new knowledge concerning fluids, electrolytes, and nutrition. This new knowledge has come about through the development of laboratory procedures which can measure changes in fluids and electrolytes. In particular, the development of the flame photometer has contributed greatly to simplification and acceleration of numerous laboratory tests which in the past were too time consuming for routine practice. Tests for sodium, potassium, blood volume, total protein, albumin-globulin ratio, etc., which were infrequently done in the past, are now everyday procedures, and can be made rapidly, frequently, and efficiently, so that they are readily available to the practicing clinician. Flame analysis is discussed on pages 414 ff.

PHYSIOLOGY OF BODY FLUIDS AND ELECTROLYTES

Body Fluid Compartments

The total body water as measured by deuterium oxide (D₂O) is approximately 60 per cent of the body weight, and is equal to about 42 liters. The body water is distributed between three fluid compartments: intravascular, interstitial, and intracellular. The first two are grouped together and called the "extracellular."

*Pages 395 to 413 have been written by Jacob J. Weinstein, B.S., M.D., F.A.C.S., Associate in Surgery, School of Medicine, George Washington University; Associate in Surgical Research, Garfield Memorial Hospital, Washington, D. C., and William C. Liu, B.S., M.D., Fellow in Surgery, School of Medicine, George Washington University.

¹Huggins, Charles: *J. Urol.* 46: 997, 1941.

²Lab. Digest 5: No. 10, March, 1942.

The plasma or "intravascular" volume constitutes 5 per cent of the body weight and is equal to 3,500 c.c. in the 70 kilogram man. This space is measured by such laboratory procedures as the Evans blue dye method, and with radioactive iodinated albumin. The Evans blue method is now a standard procedure in many hospitals, and yields much valuable information in the sick and depleted patient.*

The interstitial or the active component of the extracellular compartment makes up about 12 to 15 per cent of the body weight and is equal to approximately 8.5 to 10.5 liters. The extracellular space is measured by inulin, sucrose, or thiosulphate disappearance. The interstitial component is estimated by subtracting the plasma volume from the total extracellular body water estimates. The interstitial fluid is intimately related to the intravascular and intracellular water. Different tissues contain different amounts of interstitial fluid. For example, the kidney has the largest amount of interstitial fluid, 57 per cent, the muscle the least, 13 per cent. Other tissues have amounts varying between 24 and 49 per cent.

The intracellular compartment is the largest water compartment in the body, and is equal to 43 to 50 per cent of the total body water, or the equivalent of 30 to 35 liters of fluid.

Body water may move from one compartment to another without restraint; thus fluid exchange is in a dynamic state. Water intake is applied almost directly to the extracellular compartment, whereas normally the intracellular volume is maintained from water obtained by oxidation of food substances. The distribution of body water throughout the three compartments is associated with many mechanisms: by circulation of the blood, level of plasma protein, permeability and semipermeability of capillary and cell walls, selective permeability of the membranes and cells, and rate of solubility and diffusion of cations and anions. Therefore, water deprivation or dehydration is reflected in all compartments as is the overhydration of water overload.

Sources of Body Water

Water or fluid obtained by drinking is the major source of body water. The water contained in and derived from food also is an important source of body water. Water obtained from oxidation of foodstuffs and from oxidation of body substances burned for energy is the third source of body water. Different foodstuffs yield different amounts of water of oxidation: 1 gram of fat gives 1 gram of water; 1 gram of carbohydrate, 0.6 gram of water; 3 to 500 grams of water are derived from oxidation of organic food materials in the usual mixed diet, that is, about 12 grams of water for each 100 calories.

Functions of Body Water

Water is indispensable to life. It is constantly expended in normal activities even when the source is decreased. Water deprivation leads to depletion of all body stores, which produces dehydration and eventually death. All of the foodstuffs, nutrients, and electrolytes are in solution in water. Best

*Evans blue may be obtained from Warner-Chilcott, Laboratories Division, New York, N. Y. Full directions for use are enclosed with the dye.

and Taylor give the following physiologic properties of body water: (1) respiratory: the transportation of oxygen from the air in the lungs to the tissues and of CO_2 from the tissues to the lungs; (2) nutritive: the conveyance of food material, glucose, amino acids, and fats from the alimentary canal to the tissues; (3) excretory, removal of waste products of metabolism, uric acid, urea, creatinine, etc.; maintenance of normal water content of the tissues through the interchange of fluid between vessel walls, intercellular and intracellular spaces; (4) regulatory for body temperature: (a) specific heat—higher than any other liquid or saline; (b) high conductivity—thermal conductivity greater than any other liquids; (c) high latent heat of evaporation—more heat is required for vaporization of water than for an equivalent amount of any other fluid; (d) protective and regulatory—blood and lymph contain certain chemical substances of a complex nature, antitoxin, lysozyme, and other antibodies.

Water Loss

Body water is lost through vaporization from the skin and lungs; through excretion through the kidneys and urine; through secretion in the gastrointestinal tract. The water lost through the skin and lungs is labeled "insensible fluid loss"; that lost through the kidneys is called "sensible fluid loss" because it can be readily measured. In a healthy person in a temperate climate, the amount of water lost in the expired air is 300 to 600 c.c.; through the skin, 300 to 600 c.c.; 1,200 to 1,800 c.c. are excreted by the kidneys, and 100 c.c. are lost through the gastrointestinal tract. Thus, 600 to 1,200 c.c. are lost by the insensible route and 1,300 to 1,800 c.c. by the sensible. The insensible loss is rather constant under normal conditions. However, insensible loss is related to environmental temperature and humidity as well as to the rate of respiration, volume of respiratory exchange, and content of water inhaled and exhaled. These latter two are dependent upon the temperature and humidity of environmental air. The insensible loss of water through the skin is a reflection of gradient diffusion through the skin. If there is an increased need for heat dissipation from the body, fluid is elaborated on the surface of the sweat glands. The amount of sweat elaborated is that amount required to maintain body temperature when metabolic production of heat and positive heat balance are greater than losses by evaporation. As much as 2 to 3 liters of water may be lost at high environmental temperatures.

The insensible loss of fluid has a preferential right over the available fluid, and the remaining fluid is all that is available for the kidney and the gastrointestinal tract to lose. The kidney is the main organ for the control and maintenance of body water, but the diseased kidney cannot regulate the amount lost accurately and unless adequate amounts are present, greater losses may occur with ensuing dehydration. The reverse may also be true.

Water Disturbances

Deficit may be acute or chronic and represents a decrease of the water content of the body in relation to total solutes. Clinical signs of dehydration appear when the body water is depleted by 6 per cent. Acute dehydration

is seen in vomiting, in severe gastrointestinal diarrhea, or in dysentery. Chronic water deficits are more subacute; chronic water deficits are noted after major surgery when the intake is inadequate, when vomiting or tube drainage is not balanced by parenteral fluids, or in patients with biliary drainage through T tubes or intestinal fistulae, and in medical diseases associated with hyperpyrexia, excessive urinary diuresis, diabetic coma, and heat exhaustion.

Dehydration may be either hypertonic or hypotonic. In hypertonic dehydration, there is a greater loss of water than electrolytes, and in hypotonic, the reverse is true. Hypotonic dehydration is recognized by a decreased blood sodium, and an increase in the blood hemoglobin and protein. Clinically this form of dehydration is characterized by a feeling of lassitude, weakness, decrease in urine voided, decrease in glomerular filtration, and elevation of non-protein nitrogen. Hypotonic shock is called "medical shock" and responds better to blood plasma and electrolyte solutions.

Water excess results from greater intake than output, or when the kidneys cannot regulate water balance, as seen in anuria. Overhydration produces a decrease in the concentration of serum chloride, sodium, and other electrolytes with an increase in the extracellular space. Pure water intoxication is produced by an excessive ingestion and by the excessive infusion of distilled water in the presence of impaired kidney excretion. Overhydration may also follow sodium retention as seen in renal disease, acidosis, congestive heart failure, and after surgery when excessive amounts of sodium chloride are infused. The blood findings may be normal or show slight increases. Actually, most of the increase in electrolytes is noted in the interstitial space with its concomitant expansion.

Though we have tried for clarity and organization to separate water balance from electrolyte balance, this separation is rarely present in clinical experience and one must remember that changes in water affect all the electrolytes.

Renal Regulating Mechanisms for Water Control

The kidney plays a very important physiologic role in the control of volume and concentration of body fluids. However, this control is a complex process which involves different regulating mechanisms of the cardiovascular system, the humoral and neural control of the blood pressure, the capillary bed, and the adrenal and other endocrine glands. Renal excretion of water is practically the resultant of glomerular filtration and tubular reabsorption. The following conditions will be briefly discussed:

Passive Water Reabsorption.—

The control of the excretion of the sodium and chloride determines the volume of extracellular fluid. About 85 per cent of the glomerular filtrate is reabsorbed from the proximal renal tubules without changing osmotic pressure in the tubules. The rate of this reabsorption is proportional to the rate of the glomerular filtration and plasma concentration.

(1) Decreased passive reabsorption which leads to **increased urine volume** of medium specific gravity is produced by osmotic diuresis: i.e., low

threshold substances, such as urea, glucose, sucrose, produce diuresis by a rise in osmotic pressure; inhibition of tubular reabsorption by mercurial diuretics; impaired reabsorption of the solute in renal disease such as chronic nephritis; increased filtration rate as seen in changes of arterial pressure, renal flow, and glomerular pressure.

(2) Increased passive reabsorption which leads to **decreased urine volume** is caused by decreased filtration rate due to circulatory failure as a result of increased venous pressure and slow blood flow; excessive sodium reabsorption found in edematous states, the cause of which is unknown; decreased filtration rate noted in certain types of renal diseases such as acute nephritis, lower nephron nephrosis; active water reabsorption in the distal tubules: sodium bicarbonate and water are absorbed by processes which are more or less independent of each other and under independent regulation. The rate of the reabsorption is under the control of the antidiuretic hormone or the hypophysis. From 0 to 15 per cent of the glomerular filtrate is reabsorbed from the distal tubules.

Active Water Reabsorption.—

(1) Decreased active reabsorption leads to **increased urine volume** of low specific gravity. This is caused by hypotonicity of plasma due to excess water intake, and posterior pituitary deficiencies.

(2) Increased active reabsorption leads to a **decreased urine volume** of high specific gravity and is produced by water deprivation with hypertonicity of plasma, excess salt administration, and nonspecific stimulation of posterior pituitary by emotion, exercise, syncope, epinephrine, anesthetics, barbiturates, and morphine.

Excretion of water is impaired in the following conditions: salt depletion syndrome; adrenal insufficiency; and in some edematous states such as ascites, nephrosis, and congestive heart failure.

SODIUM

Sodium is one of the more important cations in the body fluid. It plays an essential role in the following functions:

1. Regulation of acid-base equilibrium. Sodium is the most predominantly available cation to form bicarbonate.

2. Maintenance of normal osmotic pressure in the body fluid. Sodium is the chief cation in the extracellular fluid for the control of the amount and distribution of body water.

Distribution of Sodium

The concentration of sodium in the blood plasma is from 137 to 147 meq./L., approximately eighteen times that of potassium. It has close relationship with chloride, for it exists in combination as sodium chloride.

Sodium chloride constitutes about 98 per cent of total plasma sodium and other extracellular body fluids with the exception of gastric juice in which the chloride is present with hydrogen ion instead of sodium. Sodium is primarily present in the extracellular compartment. Moore has shown that the normal

concentration of 7.5 meq./kilo. of sodium is present and 1,000 meq./ in the entire skeletal system. He further stresses the importance of the fractions of tissue sodium in cases of metabolic emergencies.

Physiology

Sodium is a chief cation in the extracellular fluids. Because of its abundant distribution in the extracellular space and enormous reabsorption from the renal tubules, it becomes essential for the function of regulation of volume and distribution of body water. Since the cell membrane is permeable to sodium, the shift of sodium from extracellular into intracellular space, and vice versa, will alter greatly the amount of sodium available to form bicarbonate, and subsequently it will result in a profound disturbance in acid-base equilibrium and water balance.

Intake and Output

Under conditions of normal gastrointestinal function, sodium is practically all absorbed from the gastrointestinal tract. The daily requirement is about 100 meq. or 4.5 grams. Most of the sodium is excreted in the urine; the other is eliminated in the gastrointestinal juice, sweat, and feces.

It is interesting to note that the normal individual has a tremendous tolerance to salt. One can handle as much as 30 to 40 grams a day, because the kidneys can excrete sodium chloride in the urine in a concentration up to 2 per cent. On the other hand, if there is excessive loss of sodium without proper replacement for some time, the body water will be depleted, with the result that the nitrogen elimination will be impaired and will give rise to retention of nonprotein nitrogen, especially urea.

Renal Regulation of Sodium

The kidney is the chief organ of normal regulation of sodium. More than 85 per cent of the amount of filtered plasma sodium is reabsorbed from the renal tubules; however, the mechanism is not well understood.

Increased excretion of sodium is seen in the following conditions: increased intake or endogenous production of sodium; increased oral or parenteral intake: an excessive intake of sodium can be well tolerated in health, as the kidneys can excrete enormous amounts of sodium in the urine even up to 2 grams per 100 c.c. of urine; excessive chloride intake, such as its acidifying salts, ammonium chloride, and calcium chloride: more sodium will be excreted in the urine along with the excess chloride; excess tissue protein breakdown releasing phosphate and sulphate which will lead to increased sodium excretion; production of ketone acid anions due to diabetic acidosis will increase sodium excretion; decreased reabsorption of sodium leads to increased excretion of sodium in the urine: mercurial diuretics definitely diminish reabsorption of sodium due to the toxic damage of mercurials to the renal tubules; osmotic diuretics as low renal threshold substances (examples, urea and glucose) will decrease tubular reabsorption as a result of change of osmotic pressure; adrenal insufficiency, such as in Addison's disease. It is well known that adrenal salt-water hormone regulates the reabsorption of sodium. In cases of Addison's disease, this hormone is inadequate and the tubular re-

absorption is impaired. Renal disease, especially chronic nephritis, is the so-called salt-losing nephritis, a condition characterized by impaired reabsorption. Increased filtration rate: renal excretion is chiefly regulated by tubular reabsorption and glomerular filtration; therefore, an increased glomerular filtration rate due to increased renal flow and pressure will subsequently increase renal excretion.

Decreased excretion of sodium is seen in decreased intake; the kidneys have remarkable power for conserving sodium. There is a tendency to decrease sodium when there is deprivation of sodium.

Increased reabsorption is seen in hyperfunction of adrenal cortex as desoxycorticosterone acetate or adrenal cortical tropic hormone: these substances have remarkable salt-retaining power to regulate the renal tubular reabsorption; in edema there is an excessive reabsorption of sodium for some reason; sex hormone administration: there has been observed that there is a marked decrease in excretion of sodium during administration of sex hormones, especially progesterone—presumably, this hormone has the same salt-retaining effect as salt or administration of the hormone of the adrenal cortex; decreased filtration rate due to elevated venous pressure, decreased renal flow and pressure, as seen in circulatory failure and congestive heart failure.

(1) Increased Plasma Sodium

When plasma sodium is above 155 meq./L., increased plasma sodium is present. It is not encountered frequently in medical practice; however, sodium excesses should be borne in mind in the following conditions:

Cushing's syndrome. Sodium is increased in plasma and is frequently associated with increased plasma chloride, decreased plasma potassium, and a tendency to alkalosis. This disease is probably due to hyperfunction of the adrenal cortex which follows an excess of adrenal tropic hormone or hyperfunction of the anterior hypophysis.

Congestive heart failure. The excretion of sodium is markedly decreased because the filtration rate is diminished. Overadministration of sodium will subsequently increase sodium level in plasma.

Administration of sex hormones because of its similar salt-retaining effect as that of adrenal cortex extracts.

(2) Decreased Plasma Sodium or Sodium Deficiency

When the plasma sodium is below 130 meq./L., decreased plasma sodium or sodium deficiency is considered to be present. It is seen in the following conditions:

Adrenal cortical insufficiency. The salt-retaining power is lessened, thus plasma sodium is decreased.

Loss of gastrointestinal secretions due to prolonged vomiting or diarrhea: a fair amount of sodium chloride bicarbonate will be lost in the gastrointestinal secretions, thus plasma sodium is decreased.

Sodium dilution as in excessive intake of salt-free or salt-poor water during excessive sweating, long-standing heart disease, liver disease, advanced

form of cancer, burns, undernourishment, anemia, etc., with marked azotemia and positive water balance.

Sodium paradox is defined by Moore as low plasma sodium due to normal weight loss as seen after injury or surgery. The plasma level is below 125 meq./L. due to negative nitrogen balance and fat oxidation. It is interesting to note that in this phenomenon there is not an actual decrease in total body sodium due to a sodium shift from extracellular space into intracellular space.

Shock. The plasma sodium is decreased; this is probably due to the sodium shift from extracellular compartment to intracellular space.

Symptomatology.—

Dehydration is the most pronounced symptom in sodium deficiency. The patient becomes very weak and apprehensive. Anorexia is a common sign. The patient may go into shock as the condition progresses. Blood pressure drops, cold sweating extremities and tachycardia are common, and coma or convulsions may develop in the later stages. Marked retention of blood urea nitrogen is seen as a result of decreased filtration rate.

Diagnosis.—

Diagnosis is based on history of dehydration with imbalanced intake and output of water and electrolytes. Loss of body weight is a very common sign in sodium deficiency as a result of negative water and nitrogen balance and extrarenal losses by vomiting, diarrhea, tube drainage, fistula, intestinal obstruction. Extrarenal loss of sodium is the most important source and cause of sodium deficiency and is the most reliable clinical guide.

Laboratory Studies.—

Laboratory studies are made to evaluate renal sodium preserving power by renal function test; to evaluate pH of blood by CO₂ combining power determinations; to evaluate osmotic pressure and oxygen-carrying capacity by determining hematocrit and serum protein.

Plasma sodium determination is now routine practice. Since the flame photometer has been developed and used as a simple method to determine sodium, it has greatly contributed to help make a correct diagnosis; however, under certain circumstances, a low plasma sodium alone does not indicate sodium deficiency. It may be due to a temporary shift of electrolytes between compartments while the total sodium content remains unchanged. (See pages 417 and 421.)

Urine sodium determinations and desoxycorticosterone acetate tests offer great contribution to differentiate between sodium deficiency due to extrarenal losses and that due to renal losses or between renal and adrenal failure. If the urine sodium is below 25 meq./L. with loss of plasma sodium, it indicates high extrarenal losses. If the urine sodium is above 35 meq./L. with low plasma sodium, it indicates high renal losses.

Desoxycorticosterone acetate serves the purpose of differentiating between renal and adrenal failure when there is high renal losses.

The test is made by giving desoxycorticosterone acetate, 3 mg. three times a day IM. If urine sodium falls in response to DOCA, this suggests renal failure.

Treatment.—

In the first place, it should be borne in mind that sodium deficiency is not a matter of sodium alone. The other electrolytes and water intake and output should be thoroughly investigated. Estimate past and current losses and approximate intake and output by proper replacement. Francis Moore recommends giving 300 meq. of sodium, 500 meq. to the patients who have low urine sodium but high extrarenal losses the first day, 100 meq. for sodium paradox. He also stresses the point that sodium and potassium are antagonistic to each other in the extracellular space. Since potassium is slightly elevated when there is sodium deficiency, sodium administration may lower the extracellular potassium level and increase diuresis. There is a tendency toward salt retention after operation because there is expansion of the extracellular compartment, especially in the first postoperative day. Sodium should be given with caution during this period. If there is salt-losing adrenal failure after operation, DOCA* and cortisone in addition to salt and water should be given. If there is sodium deficiency due to renal failure, renal function should be carefully studied and the sodium treatment given to only approximate the balance of intake and output. The urine sodium should be determined every day, and the ever common complication, uremia, should be watched for at all times.

POTASSIUM

The physiologic role played by potassium ions has been brought to the attention in medical practice in recent years. Potassium is found to be essential for the following functions: It is essential for proper functioning of cell enzymatic processes, especially carbohydrate metabolism; it helps to maintain osmotic pressure equilibrium between intracellular and extracellular fluids; it helps to maintain normal pH of body fluid; it is essential for transmission of nerve impulses.

Since the development of the flame photometer, potassium has been extensively investigated. The radioactive isotope K^{42} has been used to determine the potassium in the body. On an average, 46 meq. per kilogram of the body weight is present in males and 31.5 meq. per kilogram of the body weight in females.

Distribution of Potassium

Potassium is present in the extracellular compartment, the intracellular fluid, and in the internal secretions. However, about 98 per cent of the potassium in the body is found in the intracellular fluid.

Extracellular Distribution.—Folk and his associates gave normal serum value from 3.5 to 5.5 meq./L.

Intracellular Distribution.—Intracellular space is the major compartment for the presence of potassium, but it is the part that we know least about. Corsa measured intracellular potassium with radioactive potassium K^{42} and established the value 106 meq./L. Dean and Smith found an average of 115 meq./L. Bodansky estimated it as much as 125 meq./L. of intracellular potassium in red cells. Mudge and Vislocky estimated 150 to 160 meq./L., the

*Desoxycorticosterone acetate.

value for potassium in intracellular fluid. Darrow has given these values for various organs: skeletal muscle 155 meq./L.; cardiac muscle 142 meq./L.; liver 145 meq./L.; brain 184 meq./L.

Potassium in Internal Secretions.—The normal concentrations of potassium in internal secretions are as follows: saliva 18.5 meq./L.; gastric juice 14.0 meq./L.; hepatic bile 6.6 meq./L.; jejunal, ileo, and colonic 6.2 to 7.2 meq./L.; pancreatic secretion 4 to 5 meq./L.

Physiology of Potassium

The total potassium of the body is about 4,000 meq. Cell potassium is partially ionized. Part is bound to protein, the rest is combined with phosphate and other complexes. The unbound portion of the potassium passing through the cellular membrane is subject to shifts from one fluid compartment to another by glycogenesis. Weller and Taylor found that potassium is constantly being changed between the extracellular and intracellular spaces at the rate of 1.5 millimoles of potassium in each direction per hour. The red blood cells utilize 1 millimole of glucose for each millimole of potassium that enters the cell. It has also been observed that potassium bears a relationship with carbohydrate metabolism. Plasma potassium levels drop and potassium excretion in the urine is diminished after the administration of insulin. Fern has demonstrated that potassium is deposited intracellularly with glucose by glycogen formation in the liver.

Intake and Excretion of Potassium

The chief exogenous source of potassium is food, as meat, fruits, milk, and eggs. About 2 to 4 grams or 75 to 100 meq. are required a day. For maintaining the balance between intake and output, about 80 to 90 per cent is excreted in the urine and 10 per cent in the feces, and some is eliminated in the perspiration. The endogenous source is from tissue breakdown, water deprivation, and tissue trauma: 2.4 meq. of potassium are released when 1 gm. of nitrogen is lost as a result of tissue breakdown.

Increased potassium excretion is seen in increased oral or parenteral potassium intake; increased urine excretion due to adrenal cortical hyperactivity, adrenal cortical tropic hormone; diuresis from urea or mercurial agents, and from alteration in pH—less hydrogen ion is secreted by the tubule in exchange for sodium, and potassium has to replace it: plasma alkalosis; dilution of extracellular fluid with potassium-free fluid; water deprivation, vomiting, or diarrhea; carbohydrate metabolism, glucose with increased potassium shift; and, last, from renal damage resulting in failure to acidify urine with hydrogen ions.

Decreased excretion is seen when there is limitation of intake, diminution in filtration rate in cases of oliguria, and anuria, adrenal insufficiency, and starvation within nitrogen balance.

Extrarenal losses occur when gastrointestinal secretions and excretion are increased in prolonged and persistent vomiting or diarrhea or Wangensteen suction produces extrarenal loss of potassium. Some potassium loss is seen in sweat, but it is negligible.

Clinical Conditions of Potassium Imbalance

Hyperpotassemia.—

Hyperpotassemia is clinically present when plasma potassium exceeds 5.5 meq./L.

Causes.—Oliguria or anuria resulting in diminished excretion; excessive potassium intake; tissue breakdown—1 gm. of nitrogen lost will release 2.4 meq. potassium daily; severe water deprivation.

Symptomatology.—

Neurological: Muscular weakness, paresis, flaccid paralysis, asphyxia due to respiratory paralysis.

Cardiac: Disturbance in conduction mechanism, heart block and cardiac arrest. Electrocardiogram shows Q wave elevation, S-T depression, A-V block, auricular standstill, interventricular block, idioventricular rhythm. The lethal plasma potassium level is about 10 meq./L., and death is due to cardiac arrest.

Treatment of Hyperpotassemia.—

Parenteral administration of calcium may be helpful. Insulin and glucose administration increases potassium shift to cells. Cortisone, hydrocortisone, and corticotropic hormone increase urinary excretion. Perfusion of large bowels with saline and hypertonic glucose is found to be very satisfactory, also artificial kidney and peritoneal dialysis.

Hypopotassemia.—

Hypopotassemia is present when plasma potassium levels drop below 3.5 meq./L.

Causes of Hypopotassemia.—

Inadequate intake, excessive excretion of potassium in the urine, gastrointestinal or other body fluid secretion, expansion of extracellular space as in edema, increased transfer of potassium in the cells, alkalosis, and negative nitrogen balance are causes of hypopotassemia.

Clinical: Symptomatology.—

Generalized weakness, fish-mouth type of respirations when intercostal and diaphragmatic muscles are involved, low blood pressure, wide pulse pressure, and high venous pressure.

Cardiac: Electrocardiogram findings are prolongation of Q-T interval and inversion of the T wave.

Pathology of Hypopotassemia.—

In this condition the following may be found: myocardial necrosis, fibrosis, nonspecific inflammation of subpericardial tissues, hydropic degeneration in the proximal tubules of the kidney.

Clinical conditions, all frequently associated with hypopotassemia, are:

1. Diabetic acidosis shows elevated or normal plasma potassium level before treatment, but drops after the treatment because of the effect of insulin glycogenesis and the urinary diuresis.

2. Alkalosis caused by infantile diarrhea, or prolonged vomiting, especially gastric fluid in cases of pyloric obstruction.

3. Prolonged alimentation with potassium-free fluid.

4. Bulbar paralysis; abnormal potassium metabolism is responsible for muscular weakness.

5. Hyperadrenal cortical activity such as is seen in Cushing's disease, adrenal corticotropic hormone administration, or testosterone and estrogen therapy.

6. After major surgery, the surgical stress produces increased sodium retention and expansion of the extracellular space, but potassium is lost at a normal rate.

7. Uremia.

8. Chronic nephritis, because of damage to the tubules, resulting in poor reabsorption of potassium.

9. Bulbar poliomyelitis.

10. Ulcerative colitis.

11. Toxic doses of digitalis.

Diagnosis of Hypopotassemia.—

1. Balance studies: intake and output imbalance, excessive excretion, or drainage.

2. Clinical history and symptomatology, loss of strength, anorexia, chronic ileus, motor paralysis, muscular weakness.

3. Electrocardiogram, lengthening of Q-T interval, depression of S-T segment, lowering and broadening in inversion of T wave, appearance of U wave.

4. Biochemical analysis of plasma, lower plasma potassium level, unexplained high serum bicarbonate, unexplained low sodium in plasma.

5. Common occurrence of potassium deficiency in the following conditions: diabetic acidosis, intestinal obstruction, pyloric obstruction, prolonged vomiting and diarrhea especially in infants, prolonged parenteral administration with potassium-free fluids, periodic familial paralysis, chronic nephritis, Cushing's syndrome, dehydration, colitis, sprue, testosterone, estrogens, bulbar poliomyelitis.

Treatment of Hypopotassemia.—

Oral administration of potassium is preferable to parenteral use whenever it is possible; however, parenteral administration can be given when there is adequate urinary output, low plasma potassium level, and normal blood pressure. Potassium deficiency is not an isolated state; it usually comes along with imbalance of other electrolytes. Serial electrocardiograms should be made and plasma potassium determination and CO_2 combining power determination should be checked frequently during the treatment. Sufficient amounts of carbohydrates should be given together with potassium, as carbohydrates aid the assimilation and retention of potassium.

CHLORIDE

Chloride is an essential and important anion of extracellular fluid. It is closely associated with the sodium and potassium in body tissue fluids and their excretion. It plays an essential part in the maintenance of normal acid-base equilibrium and water balance in human metabolism.

Distribution of Chloride

Chloride is abundantly distributed in the extracellular fluids. Because of its close link with sodium, it has more or less the same distribution as sodium. The chloride content in whole blood is 350 to 390 mg. per 100 c.c. or 100 to 110 meq. per 1,000 c.c. There is a marked difference between the chloride concentration of the intracellular and extracellular compartments. There is only one-half as much plasma chloride in the red blood cells because of a relatively high concentration of protein and low water content within the red blood cells. The chloride content in gastric secretion is 40 per cent greater than that of plasma. The chloride of intestinal secretions is about 7 per cent greater than that of plasma. Hepatic bile and pancreatic juice have about 35 per cent greater chloride content than does plasma. Chloride in sweat is approximately the same as that of plasma, whereas the cerebrospinal fluid has 720 to 750 mg. of chloride per 100 c.c. in the form of sodium chloride.

Physiology

The daily intake of chloride is estimated to be 10 to 15 gm., in the form of sodium chloride. These two ions are generally ingested together and together provide for the maintenance of normal osmotic pressure, of acid-base balance, and of water balance. The elimination of chloride approximates the intake. Chloride is one of the threshold substances chiefly eliminated by way of the kidneys by glomerular filtration and tubular reabsorption. About 4 to 5 per cent is estimated as being eliminated in the sweat and only 1 per cent is excreted in the feces.

Chloride has a very close relationship with bicarbonate in the blood. There are reciprocal changes in chloride and bicarbonate concentrations which preserve the osmotic pressure and acid-base equilibrium. The integral buffering role played by chloride is called the "chloride shift," and has been investigated thoroughly during the oxygen and carbon dioxide exchange in the red blood cells. When the blood is oxygenated, the cell chloride diffuses through the cellular membrane into plasma, while bicarbonate leaves plasma and enters the erythrocytes. The same shift takes either form, cell to plasma or vice versa, when there is change of p_H in the blood. Therefore, chloride shift is the most important indicator of development of acidosis or alkalosis.

Hyperchloremia

1. Anemia.—

Because of the relative increase in the ratio of plasma to the cells, the chloride content is relatively increased. Actually, there is no absolute alteration of chloride of clinical importance.

2. Kidney Diseases.—

Blood chloride is elevated in some cases of nephrotic types of nephritis and nephrosis because the chloride excretion is diminished.

3. Urinary Obstruction.—

Urinary obstruction due to prostatic enlargement or calculi without vomiting or diarrhea produces hyperchloremia and is probably due to impaired chloride elimination in the urine.

4. Essential Hypertension.—

Hyperchloremia is not a very common feature, although it is believed that chloride or sodium retention occurs in hypertension with kidney changes.

5. Cardiac Decompensation.—

There may be normal, increased, or decreased plasma chloride in this disease; variations depend on the method of treatment and on kidney function.

6. Hyperventilation.—

Excessive amounts of CO_2 are eliminated in the expired air with hyperventilation and this produces a chloride shift from cells to plasma, thereby producing hyperchloremia.

7. Excessive Chloride Administration.—

Excessive chloride administration is usually accompanied by acidosis.

Hypochloremia**1. Loss of Gastrointestinal Secretions.—**

Chloride is the principal anion of gastric hydrochloric acid. In cases of excessive vomiting due to pyloric obstruction, pylorospasm, gastric irritation, small intestinal obstruction, uremia, and toxemia of pregnancy, hypochloremia is a common feature, as well as alkalosis.

2. Uremia and Acidosis.—

There is considerable loss of chloride due to vomiting and diarrhea. Occasionally in cases of uremia there is marked chloride shift from plasma to the cells when acidosis is produced rather than alkalosis.

3. Diabetic Acidosis.—

The hypochloremia is partly due to excessive loss of chloride in urine from prolonged diuresis, partly due to vomiting, and partly due to chloride shift into the cells from the existing acidosis, and, last, from the hyperglycemia which raises the osmotic pressure, and thus reduces the requirements for chloride.

4. Starvation.—

Lower intake of chloride and continued excretion of chloride in the urine will bring about hypochloremia.

5. Bichloride of Mercury Poisoning.—

Chloride value is very low in plasma. It is probably due to loss of chloride by vomiting and diarrhea, and impaired reabsorption of chloride from renal tubules.

6. Emphysema.—

Long-standing emphysema will bring about a chloride shift into cells by increasing carbon dioxide tension in the alveolar air.

7. Infections.—

Especially in cases of pneumonia and pulmonary tuberculosis hypochloremia is found.

8. Adrenal Cortical Insufficiency.—

There is suppression of a salt- and water-regulating hormone, which normally maintains the sodium, potassium, water content, and body fluid distribution.

9. Other Causes.—

Administration of large quantities of sodium chloride-free solution, hyperparathyroidism, and acute hepatic disease may also produce hypochloremia.

Diagnosis

1. Hypochloremia is usually accompanied by potassium or sodium deficiency, and symptoms are related to those associated with either low potassium or low sodium, or both.

2. The CO_2 combining power is elevated and plasma chloride is below 90 meq./L.

3. Uremia, acidosis, severe dehydration, pyloric obstruction, pancreatic fistula, emphysema, starvation, Addison's disease, pneumonia, and vomiting and diarrhea, if present, should make one aware of low chlorides.

4. Fantis test for chloride in urine will show a decreased excretion. It is a simple and accurate test for clinical purposes.

Fantis Test.—

Mix 10 drops of urine with 1 drop of 20% potassium chromate solution.

Add 2 to 3% silver nitrate solution, drop by drop.

Shake the tube well, until the color changes from yellow to brown.

The number of drops of silver nitrate used represents grams of sodium chloride per liter of urine.

Treatment

Patients usually respond to the administration of sodium or potassium chloride. In cases where reduction in the excretion of sodium exists, as in postoperative periods, with low plasma chloride, ammonium chloride is preferred to correct the deficit.

ACID-BASE EQUILIBRIUM

The acid-base balance is regulated by two types of mechanism. One is a physical and chemical process which is under the control of the respiratory center (carbon dioxide), the other is a physiologic balance which is governed by the excretion of acid or base electrolytes by the kidney.

Physical-Chemical Regulation

Regulation of the pH of blood is closely related to the chemistry of respiration. This regulation is accomplished by carbon dioxide which is continuously produced and available in great quantities to form carbonic acid that neutralizes the blood with alkalies by its conversion into bicarbonate. The carbon dioxide combining power with its alkali reserve therefore becomes an indication of the pH of blood. The pH of blood is the ratio of carbonic acid to bicarbonate. This ratio varies a great deal during the gaseous exchange of carbon dioxide and oxygen in the tissues and in the alveoli of the lungs. When carbon dioxide tension is high in the tissue, there is a shift of bicarbonate from the cells to plasma, while chloride enters the cells from the plasma. This chloride-bicarbonate shift is revealed when the blood is oxygenated in the alveoli of the lungs. Moreover, the respiratory center is very sensitive to variations of CO_2 tension and pH of blood. Increased CO_2 tension will result in increased length and rate of respiration with consequent increased pulmonary ventilation to remove the excess carbonic acid content (CO_2). Thus carbon dioxide plays a prominent role and perhaps the most essential part in regulation of acid-base equilibrium of body fluids.

The plasma proteins, sodium phosphate, potassium phosphate, and oxyhemoglobin are considered important blood buffers for neutrality of blood. The buffer action of phosphate is due to the ability of monosodium phosphate, which is weakly acid, and disodium phosphate, which is weakly alkaline, to adjust the normal pH of blood. Plasma proteins are buffers because of their amphoteric nature. They act as weak acids in blood. Hemoglobin is slightly acid and combines with potassium in the cells, forming salts of reduced hemoglobin and oxyhemoglobin which are more strongly acid. During the phase of gaseous exchange of CO_2 and oxygen, there is not only chloride-bicarbonate shift in the blood during the reduction and oxygenation of hemoglobin, but also oxyhemoglobin becomes more strongly acid. This reaches a level of seven times more acidity than is found in reduced hemoglobin.

Physiologic Regulation

The kidneys efficiently eliminate or conserve either acid or base during the tubular selective reabsorption of bound acids from the glomerular filtrate. The base available to form bicarbonate is the algebraic sum of sodium, potassium, calcium, and magnesium, minus chloride, phosphate, proteins, sulphate, lactate, and keto acids. The amount of base is essentially sodium and potassium versus chloride, and is regulated by the kidney. If there is excess of acid, there is an increased rate of ammonium formation with excretion by the kidneys, thereby diminishing the loss of base from the body and conserving alkali reserve. The excretion of phosphate by the kidney is another compensatory mechanism to increase elimination of acid-reacting urine and help reabsorption of a portion of the sodium. A relationship has been demonstrated under conditions which permit the kidneys to adjust body water and electrolytes when there is a deficit of one of the ions, such as sodium, potassium, and chloride. It is regarded as a biologic equilibrium: a deficit of one of these ions tends to lead to a deficit of the other.

ACIDOSIS AND ALKALOSIS

Metabolic Acidosis

Metabolic acidosis is produced by primary decrease in the concentration of base available to form bicarbonate. It may be caused by increases in fixed acids such as ketone bodies and lactic acid, or relative decrease in the total concentration of total base, such as sodium, potassium, and chloride. It is frequently seen in the following conditions:

1. **Diabetes Mellitus:** It is a constant feature in advanced cases. It is due to ketosis, dehydration, and excessive loss of base as a result of polyuria.
2. **Uremia:** The terminal stage of renal insufficiency often brings about retention of acid radicles which could not be eliminated by the kidneys.
3. **Anesthesia:** Due to diminished alkali reserve and accumulation of excessive quantities of lactic acid as a result of anoxemia and incomplete oxidation of glucose.
4. **Dehydration:** In association with loss of electrolytes.
5. **Toxemia of Pregnancy:** Probably due to slight alkali deficit.

Sodium deficiency alone is very rare and is usually accompanied by a deficit of potassium and chloride. Therefore, the patient with acidosis should be treated with replacement of potassium as well as sodium and chloride and the underlying causes.

Metabolic Alkalosis

Metabolic alkalosis is produced primarily by an increase in the sodium available to form bicarbonate in plasma, and a decrease in chloride. It is usually caused by loss of gastric juice by vomiting, diarrhea, suction drainage, excessive alkaline administration, or x-ray and radium irradiation due to decrease in serum phosphate. In cases of alkalosis, there is usually potassium deficiency besides a deficit of sodium. If sufficient water is available for renal adjustment, potassium will partially replace the intracellular deficit of potassium; however, if the deficit is not corrected promptly, the alkalosis may continue despite the replacement of sodium and chloride, because the biologic adjustment of deficits of potassium leads to maintenance of alkalosis by the kidneys.

Respiratory Acidosis

Respiratory acidosis results from primary increase in carbon dioxide tension. It is frequently seen in depression of pulmonary ventilation due to necrosis, injury to respiratory center, or paralysis of the muscles of respiration. Both acute and chronic respiratory acidosis may be produced by a disease of the lungs which leads to thickening of the alveolar walls, as bronchiectasis and emphysema, because anoxia is present when there is less oxygen diffused through the thick alveolar wall, leading to carbon dioxide accumulation. In cases of uncompensated respiratory acidosis, presumably there is no appreciable change in electrolytes.

Respiratory Alkalosis

Respiratory alkalosis is caused by primary decrease in carbon dioxide tension. It is produced by excessive pulmonary ventilation such as exercise,

fever, exposure to high external temperature, anoxia, infection of the central nervous system, tumors, and drugs. Presumably, respiratory alkalosis produces no change in body electrolytes.

METHODS OF STUDYING ACID-BASE BALANCE

Alkali Reserve

1. **Carbon Dioxide Capacity of Plasma.**—The carbon dioxide capacity of plasma is the number of c.c. of CO_2 which can be bound as bicarbonate by 100 c.c. of plasma at 0°C . and 760 mm. Hg pressure. It is expressed in volume per cent or milliequivalent. The normal values for adults vary from 55 to 80 c.c., about 10 volumes per cent lower in infants. If the value is above the normal range, it is considered alkalosis, while below it is acidosis.

2. **Plasma Bicarbonate Determination by Titration.**—Plasma bicarbonate determination by titration is more accurate than CO_2 combining power. Determination of carbonic acid in blood, H_2CO_3 content of blood determination, has no clinical application, because carbonic acid content varies in different portions of the body due to different metabolic activities.

3. **Alveolar CO_2 Tension Determination.**—Alveolar CO_2 tension determination is the indirect index of blood bicarbonate alkali reserve. The normal values by the Marriott method range from 5.2 to 5.7 volumes of CO_2 per cent, corresponding to a CO_2 tension of 40 to 50 mm. Hg. The Fridericia method of determination of alveolar tension is more accurate, about 10 per cent lower than that of the Marriott method.

4. **Determination of pH of Blood Plasma or Serum.**—The hydrogen ion concentration of plasma can be determined by colorimetric or electrometric methods, but it is of little clinical use because it can show only the abnormal values during uncompensated acidosis or alkalosis. It is not accurate during the early stage of acidosis or alkalosis, when it is compensated.

5. Other Methods.—

1. Alkali tolerance (Sellard's test), is of no clinical use.
2. Determination of urinary ammonia, normal value 0.7 gm. of ammonia daily. An increase in ammonia may be indicative of acidosis.
3. Titratable acidity of urine.
4. Determination of ketone bodies: normal values range from 1.5 to 2.5 mg. per 100 c.c., expressed as acetone; less than 1 gm., expressed as beta-hydroxybutyric acid in urine.

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ELECTROLYTE METABOLISM IN HYPERTENSION

Strict dietary limitation of sodium is effective in reducing blood pressure in some patients; and promotes clinical improvements in a considerable proportion of patients with hypertension. Both types of improvement are apparently the result of partial or complete restoration of normal sodium metabolism.

Predisposition to hypertension in some normotensive individuals is demonstrated by sodium retention seen when these subjects are submitted to abrupt and stringent sodium restriction. This effect may be governed by adrenal cortical activity.

A significant proportion of sodium retained in hypertension is apparently lodged in the intracellular compartment. Evidence for this includes the increase in extracellular potassium and magnesium seen in some hypertensive animals and human beings.

Regarding extreme dietary restriction of sodium inducing a reduction of blood pressure in certain hypertensive patients, Perera¹ states as follows: "The relationship of the sodium ion to the blood pressure levels in certain hypertensive states is now established beyond cavil." It may be the removal of sodium from the body which benefits these hypertensive patients.

The use of the strict rice diet and other low-salt diets in the treatment of patients with hypertensive vascular disease evinces the clinical acceptance of the importance of the Na ion in hypertension. However, widespread use of such restricted intakes has introduced new problems. Although not as likely as in congestive heart failure, the danger of low-salt syndrome exists when the rice dietary regimen is used in hypertension. Electrolyte imbalance can be expected in hypertensive patients with severe renal impairment, and also in approximately 5 per cent of those with undisturbed renal function (and not in failure), when on this dietary regimen. In these circumstances, examination of electrolyte metabolism in hypertension seems warranted.

Electrolytes in Experimental Hypertension

Extreme restriction of sodium in the diet with reduced renal hypertension does not appear to lower blood pressure in dogs, but produces slight reduction in rats. Potassium-poor diets in rats with renal-ligature hypertension are followed by a fall in pressure to within normal limits. High-sodium intake consistently augments all types of hypertension. Concentration of intracellular electrolytes is increased in renal hypertension. In a group of rats with severe renal hypertension, the aorta, stripped of adventitia, contained 34 per cent more water, 16 per cent more sodium, 43 per cent more potassium, and 13 per cent less magnesium per gram of fat-free solids than in the normotensive rats. Even rats which remained normotensive after kidney operation showed a 7 per cent increase in water and a 20 per cent increase in potassium.

¹Perera, G. A.: *J. Clin. Invest.* **32**: 633, 1953.

FLAME ANALYSIS

The study of the physiology of body fluids today has assumed great importance in all branches of medicine. As a result of the increased interest in studying body fluids, new instruments have been designed to simplify and accelerate laboratory analysis and research. Spectrochemical analyses are made because they are inherently faster than conventional analyses, and also because a good spectrophotometer gives a ready source of precision analyses when precision is required.

Flame photometry, while not fundamentally a new science (it was used in 1929 by Lundegardh for quantitative analysis of alkaline metals), is used today because it is the fastest of all spectrochemical methods, and gives extremely accurate results. Sodium, potassium, and calcium can be analyzed within a few minutes by using a flame instrument. This is important because speed is essential when electrolyte balance must be established in the patient. These elements can be analyzed by flame photometry without preliminary chemical separation.

In testing serum or urine by flame analysis, the specimen is diluted, placed in the flame, and read on a meter. Since samples can leach elements like sodium from ordinary glassware, the fewer times the sample is handled the less chance there is for error.

The flame photometer measures the intensity of the energy emitted by an element in a flame. Instead of a continuous spectrum produced by an emission source like a tungsten lamp (Fig. 89), an element in a flame emits lines or bands. These are the characteristic lines obtained by heating various substances in a Bunsen burner: the yellow color of sodium, the red color of the cobalt flame, and the green copper flame. The ordinary flame has a relatively low source of energy as compared with that used in a flame spectrophotometer.

The flame spectrophotometer excites many more lines. In using the instrument for quantitative analysis, the intensity of one of the spectral lines of the excited elements in the sample is compared with the intensity of the same line of a known amount of the element in a standard sample. Emission intensity is proportional to the concentration of the element.

Essentially, the flame instrument consists of a flame unit attached to a spectrophotometer. The one instrument serves for both colorimetric and flame technic. **To switch from flame to color measurements**, open the light door, hold it open with a shield, and swing the flame housing into position.

The Beckman Spectrophotometer.—The Beckman spectrophotometer utilizes an atomizer burner which makes possible accurate, sensitive flame analysis. The burner system maintains constant sampling, controlled by a regulated oxygen supply. At normal operating pressures, the sample will be consumed at a rate of 1.5 to 2.0 c.c. per minute. Since the reading can be made in a few seconds, the sample can be extremely small. The hot flame is produced by acetylene or hydrogen in combination with oxygen. This very intense flame is capable of producing numerous strong lines.

The burner capillary is cleaned by running a wire through it. No rinsing is necessary between samples; the direct aspiration will dry the capillary between samples, and with the use of a detergent like 1 per cent Sterox as a diluting agent, the surface tension

will be further decreased and the capillary will drain clear and clean. No cooling water is needed. The intense flame is far enough from the burner tip so that the burner dimensions are not altered, and the flame is so concentrated that the heat is dissipated just a few feet above the burner housing. No special ducts are needed to direct the heat away. With this intense flame temperature and the controlled burning system, the direct method of analysis is possible, instead of the internal standard system of compensating for varying flame conditions.

Fig. 89, page 214, shows a flame emission being measured. The solution containing calcium is being aspirated into the burner by the force of the oxygen. This solution is then drawn into the oxyacetylene flame and burned; the calcium atoms are excited by the heat, and they emit their spectral lines; the light is collected by a mirror within the flame housing, and is directed into the instrument.

The wave length dial is set so that the emission energy line at $554\text{ m}\mu$ is directed through the exit slit. The variable slit enables the instrument to pass varying bands of light, so that it operates at a maximum sensitivity for each element and permits the use of one dilution for the metal's analysis. The light energy evolving from the exit slit strikes the phototube, and produces a signal which is amplified, and then noted on the meter. By placing several known calcium standard solutions in the flame and recording their respective meter readings, a curve is plotted—concentration vs. meter reading—and the unknown samples can then be burned and these meter readings noted; the unknown concentrations are then read from the curves. See Fig. 121.

Indications for Flame Photometric Analysis

Indications for flame photometric analysis depend upon a knowledge of the physiology of electrolyte and water balance, which are explained on pages 395 ff. A brief summary is given here.

Milliequivalents per Liter

Milliequivalents per liter may be figured by the following formula:

$$\frac{\text{Milligrams in 100 c.c.} \times \text{valence} \times 10}{\text{Atomic weight}} = \text{meq./L.}$$

Electrolyte Balance.—

Body fluid contains inorganic salts dissociated into ions, the type and concentration of which differ from one fluid compartment to another. The concentration of these ions is best expressed in milliequivalents per liter, written meq./L. This is a unit of chemical power used in clinical medicine.

Body fluid consists chiefly of water and inorganic salts which are commonly called electrolytes. Body water is divided between the intracellular and extracellular compartments. The chemical structure of intracellular fluid differs greatly from that of extracellular fluid. Within the cells, the chief electrolytes are potassium, magnesium, and phosphate, as well as smaller quantities of bicarbonate and sulphate, and protein. Intracellular fluid also contains glycogen, amino acids, and nucleotides in quantities and combinations required by the cells to perform their specialized functions.

When electrolytes are in solution, as in body fluids, they dissociate into ions: electropositive or cat-ions,* and electronegative or an-ions.* The cations of the extracellular fluid are sodium, potassium, calcium, and magnesium. The anions are bicarbonate, chloride, phosphate, sulphate, small amounts of organic acids, and protein. The sum of the concentrations of the cations always equals that of the anions, which maintains the electrical neutrality of body fluids. Disturbances of body fluid balance occur when there is an excess or deficit of water or electrolytes or both.

The most important function of the electrolytes in the body fluids is regulation of osmotic pressure. The osmotic pressure within the various fluid compartments increases as the amount of electrolyte in the body fluids increases. A decline in electrolyte concentration reduces osmotic pressure. Early recognition of clinical manifestations of electrolyte imbalance is important. It is aided by blood and urine electrolyte determinations. These tests are made most accurately and fastest by means of a flame photometer.

At the present time, potassium is considered the most important intracellular cation, although in the future more important significant values might be found for some of the others.

Any disturbance in fluid volume is closely related to disturbance in electrolyte concentration. These conditions must be considered together in estimating replacement needs in therapy.

A normal individual daily takes in an excess of fluid and electrolyte through food and drink, whereupon fluid and electrolyte balance is maintained. Imbalances and abnormal shifts of fluid may occur as a result of shock, trauma, burns, gastric suction, starvation, diarrhea, and wound secretion, and all illnesses such as carcinoma, pneumonia, nephritis, and other similar conditions which damage tissue. In each case, losses are different in amount and nature.

Dehydration is the most prevalent problem of water balance. The condition hypotonic dehydration may result from poor patient management when the patient receives large quantities of fluid and little or no electrolyte. Electrolyte is initially lost from extracellular fluid with resultant decrease in ionic concentration in that compartment. To restore equilibrium, water moves into the cells, leaving the extracellular compartments depleted of water reserves. There is then a low concentration of sodium and chloride in the serum, the urine is dilute, and there is weakness, pallor, decreased cardiac output, and lowered blood pressure.

In excessive perspiration, high fever, and in kidney disease associated with polyuria when inadequate water is consumed, water is lost from extracellular spaces and the electrolyte concentration increases. Water then moves from the cells to restore osmotic equilibrium. There is no change in the pulse or the blood pressure.

Since water is freely diffusible across cell barriers, its movement is determined by changes in concentration of the electrolytes on either side, principally sodium and potassium. Changes in extracellular electrolyte concen-

*Cathode ; anode.

tration occur most commonly and are therefore most often the basis for these shifts of water. They are the result of other significant changes occurring in the body.

Sodium.—

A deficit in sodium may result from lack of sodium intake, extreme losses due to excessive perspiration, excessive gastrointestinal discharge, or inordinate losses in the urine due to an adrenocortical insufficiency which affects the kidney's reabsorptive function.

Sodium deficiency is rapidly reflected in the disappearance of both sodium and chloride from the urine. Reduced glomerular filtration causes the serum urea nitrogen to rise. Urinary chloride is low or absent. Excessive sodium in the tissues will hold water and cause tissue edema, which may or may not be indicated in a plasma sodium determination. (See pages 399 ff.)

Chloride.—

Chloride is the primary anion of extracellular fluid and for balance study purposes may be considered an extracellular electrolyte. Chloride deficiency is usually found in cases of either potassium or sodium depletion and symptoms are related to those associated with hypopotassemia and hyponatremia. The serum chloride is below 90 meq./L. in alkalosis, and the CO_2 combining power is usually elevated. (See pages 407 ff.)

Potassium.—

Potassium is the chief intracellular cation. In some instances the body may lose half its cellular potassium before the condition is reflected in the serum potassium level. It is lost in any case where there is cell breakdown. When the cells disintegrate, potassium moves into the extracellular fluid and is rapidly excreted because it is not sufficiently reabsorbed by the kidney. Alkalosis is the most important condition contributing to the loss of intracellular potassium.

In surgical situations, the principal causes for hypopotassemia are the loss of potassium through inadequate potassium intake prior to and following surgery, stress, vomiting, gastric and intestinal suction, diarrhea, and fistulae. Hypopotassemia itself increases gastric secretions so that more electrolytes are lost through these secretions. Many factors interfere with potassium balance: abnormalities of kidney function; gastrointestinal losses; cell breakdown as in starvation, dehydration, trauma, or disease; excessive administration of potassium-free fluids, particularly glucose and saline; increased excretion such as follows a course of ACTH or cortisone therapy; familial periodic paralysis; diabetic acidosis; and excessive administration of sodium during treatment or prevention of dehydration. There are many electrocardiographic changes indicative of potassium imbalance. (See pages 403 ff.)

Magnesium.—

Magnesium is present in all body cells and body fluids, but chiefly in muscle and bone. It appears to be closely related physiologically to sodium in that secretion of both seems to be similarly controlled. Of the ingested magnesium, 40 per cent is found to be excreted in the urine, the balance being found in the feces. Magnesium is the co-factor with thiamine in intermediate

carbohydrate metabolism. A definite correlation between the severity of diabetes and the magnesium content of the blood serum has been observed. A deficit of magnesium is indicated by hyperexcitability progressing to tetany.

Calcium.—

Calcium may become fixed to tissue exudates, as in burns or infections, or to fatty acids, as in acute pancreatitis, and thus be lost. When acute or chronic calcium deficits occur, the mobilization of calcium from the bones does not keep pace with loss and the serum calcium level may drop.

Calcium deficit may result from acute pancreatitis, generalized peritonitis, massive infection of subcutaneous tissue, burns, and in duodenal, pancreatic, and small intestinal fistulae. Calcium deficiency is corrected by the administration of calcium chloride or calcium gluconate.

Phosphate.—

Phosphate is an important and integral part of intracellular metabolism. Inorganic phosphate moves with potassium into the cell in conjunction with the phosphorylation of glucose. The fact that there is a close linkage between potassium, phosphorus, and magnesium has led Butler to emphasize the necessity for a repair solution to contain all these ions in the treatment of diabetic acidosis.

The Beckman Flame Photometer

Technic of Using the Flame Photometer.—

1. Before operating the apparatus, first locate all controls:
 - (a) On the left-hand side, looking at the front, is the power switch (red light when turned on) and the lamp (white light when turned on).
 - (b) On the front of the apparatus, from left to right, are:
 - (1) Sensitivity knob
 - (2) Dark current knob.
 - (3) Wave length control knob.
 - (4) Slit control knob.
 - (5) Switch used to turn the phototubes on and off.
 - (c) To the extreme right is a screw type plate in the side of the apparatus in which the phototubes are placed.
2. In the rear of the apparatus is the burner which is used in flame photometry and the oxygen and acetylene gauges.
3. Other attachments are two phototubes.
 - (a) One is frosted and is called the "red" tube. It is used for the serum potassium test.
 - (b) The other is clear. It is called the "blue" tube. It is used for making a serum sodium test and also for spectrophotometry.
 - (c) Attached to these tubes are small objects known as MEGS (megohms), which are properly labeled "500" (used for spectrophotometry) and "10,000 MEGS" (used for flame).
4. There are also 4 small square cuvettes with 1 large cuvette holder containing 4 spaces for spectrophotometry.

Preparation of the Apparatus.—

Prepare the instrument by putting the proper phototube and resistor in place:

DETERMINATION	PHOTOTUBE	RESISTOR	WAVE LENGTH
Sodium	blue	10,000 megohm	589 m μ
Potassium	red	10,000 megohm	768 m μ

Turn the "red" light power switch on to allow the apparatus to warm up for 10 to 20 minutes.

Lighting the Flame.—

Be sure the gas and acetylene regulators on the tank and in the instrument control panel are off. This can be checked by turning the handles counterclockwise until they turn very freely and exert no pressure on the regulator diaphragms.

Always turn the oxygen on first and off last.

Slowly open the valve on the oxygen tank, making sure to check the gauges so that the tanks contain a sufficient amount of oxygen and acetylene to raise the needle to approximately 20 psi.

Turn the valve on the acetylene tank to approximately 10 psi.

Open the oxygen regulator on the control panel until the gauge reads about 10 psi. Slowly open the fuel regulator on the control panel to about 3 to 5 psi, while holding the match next to and just below the tip of the burner.

If there is any difficulty in igniting the gas, turn the oxygen pressure down to between 5 and 10 on the control panel.

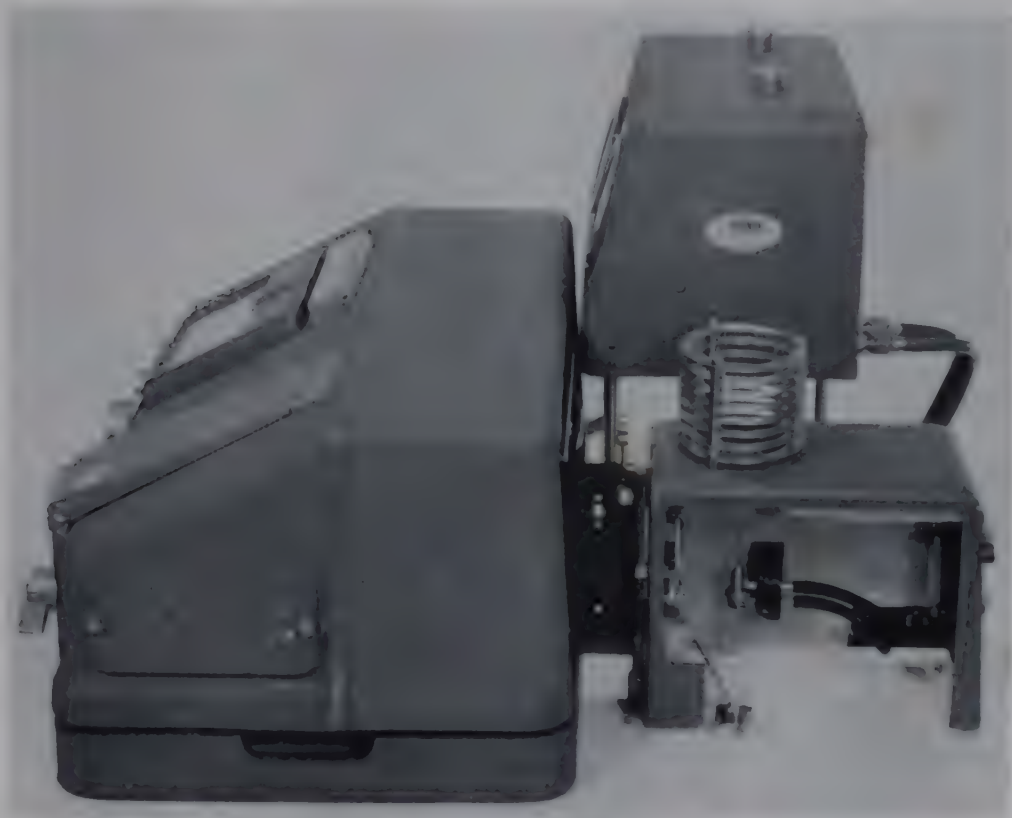


Fig. 120.—The Beckman Model B spectrophotometer with flame attachment. (Courtesy Beckman Instruments, South Pasadena, Calif.)

Adjust the fuel with the control panel regulator to provide a flame with a visibly blue portion about 2 or 3 inches high.

After the best operating pressures have been determined, it is well to mark them with a China marking pencil on the oxygen and acetylene regulator dials.

WARNING: "Although the flame radiates little heat and is quite small, it has so high a temperature that contact of the flesh with even the outer edge of the flame will instantly produce a third-degree burn. Except when lighting the burner, keep the hands completely out of the housing whenever the flame is burning."

Technic of the Actual Test.—

Turn the sensitivity switch to position 4 and balance the meter needle at "0" with the dark-current control.

Set the wave length close to the wave length required for the determination.

Place a portion of the high standard (page 422) in a Desiccated 5 c.c. beaker. Put the beaker in the apparatus under the capillary, and open the shutter. (This is called "burning the high standard.")

Readjust the wave length for peak response by carefully rotating the wave length selector a little to each side of the wave length setting until a maximum reading is obtained on the meter.

For highest accuracy, always adjust the wave length scale for peak response on the standard at each new wave length to insure exact wave length setting.

By adjusting the slit width, set the sodium high standard to read 75 per cent T (transmission) or potassium to read 61 per cent T.

After making these adjustments, lower the standard from the capillary.

Turn the shutter switch to off.

Recheck the dark current.

Reposition the most concentrated standard under the capillary.



Fig. 121.—Using the Beckman Model B spectrophotometer, with flame attachment. (Courtesy Beckman Instruments, South Pasadena, Calif.)

Open the shutter.

Read the meter.

If necessary, adjust to 75 per cent or 61 per cent T.

Read the medium and low standards (page 422). (Burn the medium and low standards.)

Read the unknown sample. (Burn the unknown sample.)

Repeat the whole series of readings, reading the high standard 3 times consecutively, medium 3 times, low 3 times, and unknown 3 times.

Speed in obtaining readings is important.

Using a curve plotted from the three standards, figure the meq./L. of potassium or sodium, etc.

It is best to burn the three standards each time an unknown is burned.

Adjust the high standard to the same transmission as the high on the established calibration curve.

Potassium tests are run the same as sodium after the wave length and phototube are changed. By setting the high standard to read 61 per cent transmission, potassium values can be determined directly from the scale reading by dividing by 10, since potassium follows Beer's law.

After running a determination, aspirate distilled water through the burner to flush it.

Shutting Off the Apparatus.—

To shut off the instrument at the end of each day, turn off the acetylene regulator on the tank. After both regulator dials read "0," turn off the regulator on the control panel. This procedure eliminates gas pressure in the connecting hose.

Turn off the oxygen regulator on the tank after both regulator dials read "0," then turn off the oxygen regulator on the control panel.

ALWAYS turn the oxygen on first and off last.

Keep the instrument covered when not in use.

CAUTION: Oxygen and acetylene cylinders should be placed away from open flames and should always be checked for leaks. Be sure that all oxygen equipment (tank, regulator, hoses, connectors, etc.) is free from any oil or grease. Oxygen under pressure will react violently with oil or grease.

Glassware.—

Utmost cleanliness is required. It is recommended that glassware, special for flame photometry, be used. Cleaning may be accomplished in the usual manner, but the glassware should be washed in a nonionic detergent and rinsed in a distilled water of the same quality as is used for preparing standard solutions. All glassware used for this work should be Desiccated.

The glassware should be handled in such manner as to keep grease and finger contamination away from the contained solutions.

Polyethylene bottles should be used for storing the standard solutions.

TESTS FOR SODIUM

Sodium is probably present in whole blood entirely in the form of the sodium ion, over 90 per cent of the total blood sodium being present in the plasma. Plasma or serum may be used for analysis. If plasma is used, the blood must be taken in a sodium-free anticoagulant. Take care not to cause transfer of water and electrolytes between cells and plasma due to changes in blood gas content. It is well to make this test as soon after withdrawal of the blood as possible.

The best method for testing for sodium is by means of the flame photometer. Because of the relatively small variations which are of clinical significance, great care must be used in precision analysis of the specimen.

(1) Determination of Sodium and Potassium Using the Flame Photometer

Principle.—

The specimen is diluted and introduced in the form of a fine continuous spray into a nonluminous gas flame. The sodium ions produce characteristic flame spectra. The emitted light characteristic of this ion is isolated and it then excites a photoelectric cell, the response of which is measured on a special meter. The meter reading is a measure of the concentration of the sodium ion. The concentration of sodium in the specimen is obtained by using a calibration curve established by analysis of a series of standard solutions of sodium ions.

Preparation of the Specimen.—

Using sterile technic, obtain 5 c.c. of blood by venepuncture.

Place in a sterile test tube and allow to clot.

Loosen the clot with a wooden applicator, centrifuge, and withdraw the serum from the clot as soon as possible. If the blood remains on the clot for any length of time, or if there is hemolysis, the test will show false high potassium readings.

Using a 1 c.c. volumetric pipette, place 1 c.c. of the serum in a 50 c.c. volumetric glass-stoppered flask containing distilled water, and dilute to 50 c.c. with distilled water.

Invert several times for thorough mixing.

Use this diluted serum for sodium and potassium determinations.

Reagents.—

Standard Sodium-Potassium Solution.—

This standard solution can be obtained from “Harleco,”* or it can be prepared in the laboratory.

Sodium chloride -----8.353 gm.

Potassium chloride -----0.380 gm.

Dissolve in distilled water in a liter volumetric flask, and dilute to 1 liter with distilled water.

Solution equivalents per c.c.:

Sodium -----142.9 meq./L.

Potassium ----- 5.1 meq./L.

The value of the solutions in meq./L. is obtained as follows:

meq./L. sodium = $\frac{\text{mg. \%} \times 10 \times \text{valence}}{\text{atomic weight}}$

or $\frac{835.3 \times 10 \times 1}{58.45} = 142.9.$

meq./L. potassium = $\frac{38 \times 10 \times 1}{74.55} = 5.1.$

Working Standards.—

Prepare three working standard solutions (see Table 28) from the above stock solution. These solutions must be stored in polyethylene bottles to prevent any interaction between the standard and the container.

TABLE 28

STANDARD	DILUTION	ACTUAL CONCENTRATION		EQUIVALENT CONCENTRATION IN 50 C.C.	
		SODIUM	POTASSIUM	SODIUM	POTASSIUM
Low	0.8 c.c. stock to 50 c.c. with distilled water	2.29	0.082	114.3	4.1
Medium	1 c.c. stock to 50 c.c.	2.86	0.102	142.9	5.1
High	1.2 c.c. stock to 50 c.c.	3.43	0.122	171.5	6.1

Calculations are simplified by working with equivalent concentrations since the serum is diluted 1:50 also.

Technic.—

Prepare the apparatus and carry out the technic as outlined on pages 418 and 419.

Use the blue clear tube for sodium determinations and the red frosted tube for potassium.

Use the 10,000 MEG for the flame.

Calculation.—

Calculated as directed on page 420 from a curve made when calibrating the instrument for sodium. Since potassium follows Beer’s law, potassium values can be determined directly from the scale reading by setting the high standard to read 61 per cent transmission and dividing the scale reading by 10.

Normal.—The normal sodium by this method is 138 to 145 meq./L. The normal potassium by this method is 3.8 to 5.0 meq./L.

*Distributed by the Scientific Products Division, American Hospital Supply Corporation, Evanston, Ill.

Example.—

SODIUM:	FIRST READING	SECOND READING	THIRD READING	AVERAGE READING	DILUTED IN E/L VALUE
High standard balanced at 75%	75.0	75.0	75.0	75.0	3.430 meq./L.
Middle standard readings	59.1	59.4	58.9	59.1	2.858
Low standard readings	47.3	47.7	47.4	47.5	2.286
Unknown diluted 1:50 readings	53.6	53.6	53.2	53.5	

Calculation: Unknown average reading is 53.5%, which lies between the low and middle standards. The difference between the low and middle standards is

	% TRANSMITTANCE	MEQ./L.
Middle	59.1	142.9
Low	-47.5	-114.3
	<u>11.6%</u>	<u>28.6 meq./L.</u>

The number of meq./L. per 1 scale division is $\frac{28.6}{11.6} = 2.46$.

Unknown read 6.0 scale divisions above the low standard. Therefore the unknown = $(6 \times 2.46) + 114.3 = 129.0$ meq./L.

(2) Titration Method of Weinbach***Reagents.—****20% Trichloroacetic Acid.—**

Dissolve 20 gm. trichloroacetic acid in distilled water and dilute to 100 c.c. with distilled water.

Uranyl-Zinc Acetate Reagent.—**Solution A:**

Dissolve 77 gm. uranyl acetate ($2H_2O$)

and 14 c.c. of glacial acetic

in 400 c.c. of distilled water by stirring and heating on a steam bath.

Dilute to 500 c.c. in a volumetric flask.

Solution B:

Dissolve 231 gm. of zinc acetate ($3H_2O$)

and 7 c.c. of glacial acetic acid in distilled water as for solution A, and dilute in a volumetric flask to 500 c.c. with distilled water.

Mix the two solutions while hot, allow to stand at least 24 hours, and filter.

95% Alcohol.—**Acetone Wash Reagent.—**

Add 15 c.c. of the uranyl zinc acetate reagent

to 1 c.c. of approximately 5% sodium chloride.

Add about 5 c.c. of 95% alcohol in small portions.

Filter with suction and wash the precipitate with 4 or 5 small portions of 95% alcohol and then with 4 or 5 small portions of ether, sucking dry after each addition of alcohol or ether.

Add this amount of the triple salt to 1,000 c.c. of acetone.

Shake and let stand overnight. Filter.

Standard Sodium Solution.—

Dissolve exactly 1 gm. of c.p. sodium in distilled water in a liter volumetric flask and dilute to 1,000 c.c. with distilled water.

Each c.c. contains 0.393 mg. of sodium.

1% Phenolphthalein Solution.—See page 1199.**0.02 N Sodium Hydroxide Solution.—**

Refer to page 25 for method of making normal solutions.

Technic.—

Place 1 c.c. of whole blood or cells (or 2 c.c. of serum or plasma) in a small flask.

Add 7 c.c. of distilled water (6 c.c. if serum or plasma is used).

Shake. Let stand until hemolysis is complete.

*J. Biol. Chem. 110: 95, 1935.

Add rapidly, with shaking, 2 c.c. of 20% trichloroacetic acid, making a total of 10 volumes. Smaller quantities may be used of the specimen and the various reagents provided the proportions are kept the same. As little as 0.1 c.c. of whole blood or 0.2 c.c. of serum or plasma may be used.

Mix. Let stand for 10 minutes.

Filter through ashless filter paper or centrifuge if the specimen is small.

Transfer 1 c.c. of the whole blood filtrate (or 0.5 c.c. of the serum or plasma filtrate) to a 15 c.c. centrifuge tube.

Add 5 c.c. of uranyl zinc acetate reagent.

From a 1 c.c. graduated pipette, add 0.3 c.c. of 95% alcohol.

Let stand for 5 minutes.

Add 0.3 c.c. of alcohol and let stand for a few minutes.

Repeat this procedure, without greatly disturbing the precipitate, until 2.1 c.c. of alcohol have been added.

The entire process of precipitation should require about 30 minutes.

Centrifuge, decant, and drain by inversion on a pad of filter paper.

Wipe the mouth of the tube with a cloth.

Wash the precipitate once by blowing in 10 c.c. of acetone wash reagent.

Centrifuge, decant, and drain on filter paper.

Wipe the mouth of the tube.

The precipitate is readily soluble in water.

Transfer the precipitate quantitatively to a 100 c.c. Erlenmeyer flask by blowing in three or four 5 c.c. portions of distilled water which have been recently boiled and cooled to drive off dissolved carbon dioxide.

Add approximately 50 c.c. of distilled water free from carbon dioxide.

Add 0.5 c.c. of 1% phenolphthalein solution.

Titrate with 0.02 N sodium hydroxide to a just barely perceptible pink color, using a microburet graduated in 0.02 c.c.

Blank: Run a blank determination to establish the amount of 0.02 N sodium hydroxide which will just give the end point with distilled water.

Determine the sodium content on a 0.5 c.c. portion of the standard sodium solution to standardize the procedure and to facilitate determining the end point.

Calculation.—

$$(\text{Titration} - \text{Blank}) \times 0.0575 \times \frac{100}{0.1} = \text{mg. sodium per 100 c.c. of blood or serum.}$$

To convert mg. % of sodium to meq./L., divide by 2.3.

The normal range of serum or plasma sodium is approximately 300 to 330 mg. per 100 c.c., or 130 to 143 meq. L. The sodium content of the erythrocytes is much less, from 4 to 16 meq./L. Whole blood sodium determinations are not recommended, since little of clinical value can be learned by such determinations. The test should be made on serum or plasma.

Explanation of Calculation: 8 moles of NaOH are required for each mole of Na. Each c.c. of 0.02 N NaOH is thus equivalent to $\frac{0.02 \times 23}{8}$ or 0.0575 mg. of Na in the sample.

TESTS FOR POTASSIUM

Potassium by Use of the Flame Photometer.—This is described on page 421. It is the recommended procedure.

Potassium, Method of Strauss¹

Potassium is precipitated as the thorium salt. After washing, the potassium is precipitated as the chloroplatinate. Potassium iodide is added, and the salt is converted into wine-colored iodoplatinate which is titrated with thiosulphate.

¹Strauss, M. B.: *J. Biol. Chem.* 118: 331, 1937.

agents.—

4 Normal Sulphuric Acid.—

Add 112 c.c. of concentrated sulphuric acid, c.p., to about 500 c.c. distilled water in a liter volumetric flask, cooling the flask under running tap water.

Dilute to 1000 c.c. with distilled water when cool.

10 per cent Thorium Nitrate [Th(NO₃)₄].—

Dissolve 10 gm. thorium nitrate in enough distilled water to make a total of 100 c.c.

0.1 per cent Phosphoric Acid (H₃PO₄).—

Dilute 1 c.c. of 85% phosphoric acid to 850 c.c. with distilled water.

Chloroplatinic Acid Containing 10 Per Cent Platinum.—

Dissolve 26.5 gm. of chloroplatinic acid (H₂PtCl₆·6H₂O) in distilled water, and dilute to 100 c.c. with distilled water.

Absolute Ethyl Alcohol Saturated With Potassium Chloroplatinate.—

Shake up the alcohol with a small quantity of the salt to obtain the saturated solution. Filter before use.

10 per cent Potassium Chloride Saturated With Potassium Chloroplatinate Solution.—

Dissolve 10 gm. potassium chloride in enough saturated potassium chloroplatinate solution to make 100 c.c. Filter before use.

2 N Potassium Iodide.—

Dissolve 33.2 gm. potassium iodide (KI) in enough distilled water to make a total of 100 c.c.

N/100 Sodium Thiosulphate Solution.—(See page 27.)

Dilute 10 c.c. of N/10 sodium thiosulphate solution to 100 c.c. in a 100 c.c. volumetric flask, and standardize against potassium biniodate.

Equipment.—

Muffle furnace that can be regulated to 750° C.

Platinum crucible or silica beaker of 15 c.c. capacity.

Shohl microfilter made by dropping a glass bead into a one-inch funnel, and making a mat of fine grained asbestos about 1/2 inch thick packed over the bead.

Technic.—

Place in silica beaker or platinum crucible

1 c.c. of serum

and 2 drops of 4 N sulphuric acid. Mix well.

Add 1 c.c. thorium nitrate and mix by careful rotation of beaker.

Place on steam bath and evaporate contents to approximate dryness (about 20 to 30 minutes).

Using an electric muffle furnace, preheated to 750° C., place the beaker on the outer door, and gradually, taking about five minutes for the procedure, introduce the beaker into the furnace. Now close the oven door, and keep at 750° C. for 10 minutes.

To a snow-white dish add 1 c.c. of the 0.1% phosphoric acid and mix thoroughly with a glass rod.

Add 5 c.c. of 0.1% phosphoric acid and mix thoroughly.

Transfer quantitatively to a clean dry 15 c.c. centrifuge tube and centrifuge.

Decant the clear supernatant fluid into another tube.

Pipette 5 c.c. aliquot into a 50 c.c. Pyrex beaker and evaporate to dryness on a steam bath.

To this, add 0.3 c.c. chloroplatinic acid and mix.

Add 5 c.c. absolute alcohol and let stand 20 minutes.

Transfer to Shohl microfilter on a suction flask containing a test tube to collect excess platinum solution for recovery of the platinum.

Wash the beaker and filter with four 2 c.c. portions of absolute alcohol saturated with potassium chloroplatinate, and pass through the filter. Now stop the suction and replace catch test tube.

Wash the beaker with four 2 c.c. portions of 10 per cent potassium chloride saturated with potassium chloroplatinate.

Remove the funnel containing the precipitate from the filtering apparatus and invert over the 50 c.c. beaker used previously.

Return the precipitate with the asbestos to the beaker by inserting a small glass rod in the stem of the funnel.

Wash the sides of the funnel with 1 to 2 c.c. hot water and 1 c.c. of 2N potassium iodide.

Heat the mixture in the water bath at 65° C. for 15 minutes.

Titrate while still hot, without removal of asbestos, with N/100 sodium thiosulphate in a microburet.

The end point of this reaction is a lemon yellow color free from red.

In all cases, run a blank determination of this whole procedure with water as an unknown solution.

Calculation.—

$$\frac{10 (A - B)}{V} = \text{milliequivalents of potassium per liter.}$$

A = c.c. N/100 thiosulphate used for titration of unknown.

B = c.c. N/100 thiosulphate used for blank.

V = 0.83 c.c. when 5 c.c. aliquot is analyzed.

$$\frac{39.1 (A - B)}{V} = \text{mg. of potassium per 100 c.c.}$$

Normal.—16 to 22 mg. of potassium. To convert to meq./L., refer to page 415.

Potassium in Blood Serum

Photoelectric Method of Looney and Dyer¹

Reagents.—

1.5% Sodium Tungstate Solution.—

Dissolve 1.5 gm. sodium tungstate, reagent grade, in enough distilled water to make 100 c.c. solution.

2.5% Copper Sulphate Solution.—

Dissolve 2.5 gm. copper sulphate in distilled water and dilute to 100 c.c. with distilled water.

2.5% Silver Nitrate Solution.—

Dissolve 2.5 gm. silver nitrate, c.p., in distilled water and dilute to 100 c.c. with distilled water.

95% Alcohol.—

0.2 N (approximately) Sodium Hydroxide Solution.—

Dilute 20 c.c. normal sodium hydroxide to 100 c.c. with distilled water.

40% Silver Nitrate Solution.—

Dissolve 40 gm. silver nitrate, c.p., in distilled water and dilute to 100 c.c. with distilled water.

Sodium Cobaltinitrite Solution.²—

See page 428.

Filter each time before using. (Stable one month.)

Cobaltinitrite Reagent.—

To 20 c.c. of filtered sodium cobaltinitrite solution, add

1 c.c. of 40% silver nitrate.

Shake well and filter to remove trace of precipitate which is undissolved.

¹Looney, J. M., and Dyer, Cora G.: J. Lab. & Clin. Med., 28: 3, 355-363, Dec., 1942.

²Harris, J. E.: J. Biol. Chem. 136: 619, 1940.

Sulfanilamide Reagent.—

Dissolve 0.5 gm. sulfanilamide in enough 30% acetic acid to make 100 c.c. of solution. This must be prepared weekly.

Coupler Reagent.—

Dissolve 0.1 gm. N-1 naphthylethylenediamine dihydrochloride in enough 30% acetic acid to make 100 c.c. solution. This must be prepared fresh weekly.

Wash Reagent.—

Alcohol-----2 volumes.
 Ether-----1 volume.
 Water-----1 volume.

Technic.—

All glassware must be kept scrupulously clean. It is best to have a set of glassware for use in potassium determinations only. Presence of ammonia will give falsely high results. Ammonia fumes must be avoided and tubes must be capped at all times when not in manipulation. The silver concentration is important, since an excess of silver as well as low temperatures during the cobaltinitrite precipitation will give long needlelike crystals of silver nitrite which interfere with the results.

(A) Precipitation of Protein and Chlorides.—

Place in a clean test tube

0.5 c.c. serum

7.0 c.c. distilled water

1.0 c.c. of 1.5% sodium tungstate. Mix and add

1.0 c.c. of 2.5% copper sulphate.

Stopper and shake well. Then add

0.5 c.c. of 2.5% silver nitrate.

Re-stopper and shake again.

(B) Precipitation of Potassium with Silver Cobaltinitrite.—

In a clean 15 c.c. graduated centrifuge tube, place

3 c.c. of the protein chloride-free filtrate

1 c.c. of 95% alcohol, and

1 c.c. of distilled water.

Place in a water bath at 18° to 22° C. for five minutes.

Add 2 c.c. of silver cobaltinitrite reagent, cap, and mix by tapping, not by inversion, and replace in the water bath for 2 hours.

Centrifuge for 15 minutes at 2800 r.p.m.

(C) Washing the Precipitate.—

With a capillary pipette remove the supernatant fluid to the 0.2 c.c. mark.

Wash with 7 c.c. of wash reagent, rinsing down the sides and disturbing the precipitate as little as possible. If the tube is slanted, the remaining 0.2 c.c. of fluid will mix with the wash solution.

Centrifuge for 15 minutes, decant, invert, and drain over filter paper for 5 minutes.

Wipe the mouth of the tube.

Repeat this washing and draining twice more.

After this stage, and only at this point, the procedure may be interrupted and the tubes allowed to stand overnight if one drop of distilled water is added to each tube to prevent the precipitate from drying.

(D) Digestion.—

Add 10 c.c. of approximately 0.2 N sodium hydroxide, breaking up the precipitate if possible.

Heat in a boiling water bath for 10 minutes. A black precipitate remains.

Cool, make up to 10 c.c. with distilled water, stopper, and mix. Replace the stopper with centrifuge cap and centrifuge for 5 to 10 minutes.

(E) Colorimetry.—

In a 100 c.c. volumetric flask place

2 c.c. of supernatant fluid from (D)

5 c.c. of distilled water
1 c.c. of 50% hydrochloric acid
and 2 c.c. of 0.5% sulfanilamide.

Mix and let stand for 3 minutes, then add 1 c.c. of a 0.1% N-1 naphthylethylenediamine dihydrochloride (coupler reagent).

Dilute to volume and read after 5 minutes.

(F) Calculation.—

Prepare a standard curve by carrying out the foregoing procedure on standard potassium sulphate solutions containing from 0.01 up to 0.12 mg. of potassium per sample. Use a photoelectric colorimeter against a water blank, using a green filter having maximum transmission at 5,200 A.U. As only one-fifth of the digested cobaltinitrite is taken for the final colorimetric reading, the absolute value of the potassium determined ranges from 0.002 to 0.010 mg.

The color produced follows Beer's law up to a concentration of 0.01 mg. of potassium in the final solution, which would correspond to a serum potassium value of 33.3 mg. per 100 c.c. of blood.

Normal potassium = 16-22 mg. per 100 c.c. serum. To convert to meq./L., refer to page 415.

Method of Kramer and Tisdall¹

Reagents.—

Sodium Cobalt-Nitrite Reagent.—

Solution A. Dissolve 25 grams of cobalt nitrate crystals (J. T. Baker) in 50 c.c. of distilled water. When the crystals have dissolved add 12.5 c.c. glacial acetic acid.

Solution B. Dissolve 120 grams of sodium nitrite (potassium-free) (Merck) in 180 c.c. of distilled water. The total volume of this solution is about 220 c.c.

To all of solution A, add 210 c.c. of solution B. An evolution of nitric oxide occurs at once. Draw air through the solution until all of the gas has been removed. This reagent has a pH of 5.7. Place in the refrigerator and filter each time before use. It is stable for at least a month.

Approximately 4 N Sulphuric Acid.—

Dilute 11.2 c.c. of concentrated sulphuric acid to 100 c.c. with distilled water.

0.02 N Potassium Permanganate.—

Dilute N or 0.1 N potassium permanganate to the proper strength. (See p. 441 for N/10 potassium permanganate.) Standardize against the 0.01 N sodium oxalate before each series of determinations.

0.01 N Sodium Oxalate.—

Dilute 0.1 N sodium oxalate 1 part up to 10 parts. The 0.1 N solution may be made by dissolving 6.7 grams of sodium oxalate ($\text{Na}_2\text{C}_2\text{O}_4$) in enough water to make one liter using 5 c.c. of concentrated sulphuric acid as an aid in getting the oxalate in solution.

Technic.—

Collect the blood by venous puncture, using a chemically clean and sterile syringe and needle. Separate the serum from the clot as soon as possible and make the determination on the serum.

Pipette 1 c.c. of serum into a 15 c.c. graduated centrifuge tube.

Add, drop by drop, 2 c.c. of the sodium cobalt-nitrite reagent.

Mix the contents of the tube thoroughly.

Set the tube aside for 45 minutes.

Add 2 c.c. of distilled water and again mix the contents of the tube.

Centrifuge at 1400 revolutions per minute for 30 minutes.

Syphon all but 0.3 c.c. of the supernatant fluid by the use of a syphon tube, the lower end of which is curved so that the opening is directed upward. Do not disturb the sediment.

¹Kramer, B., and Tisdall, F. F.: J. Biol. Chem. 46: 339, 1921.

Allow 5 c.c. of distilled water to run down the side of the tube and allow it to mix with the residual reagent. Disturb the precipitate as little as possible.

Centrifuge for 5 minutes at 1400 revolutions per minute.

Repeat three times so that the precipitate is washed four times in all. The supernatant liquid should be perfectly clear after the last washing.

Syphon off the supernatant liquid.

Add an excess of 0.02 N potassium permanganate.

Add 1 c.c. of approximately 4 N sulphuric acid.

Mix the precipitate with the fluid with a glass rod.

Heat the sample in a boiling water bath until no further change in color can be observed. This should not exceed $1\frac{1}{2}$ minutes.

Add 2 c.c. of 0.01 N sodium oxalate, sufficient to decolorize the solution completely. Determine the excess oxalate by titrating to a definite pink color with 0.02 N potassium permanganate.

Calculation.—

The total number of c.c. of 0.01 N potassium permanganate required to oxidize the potassium cobalt-nitrite $\times 7.1 =$ mg. potassium in 100 c.c. of serum. One c.c. of 0.01 N potassium permanganate will oxidize a quantity of potassium cobalt-nitrite corresponding to 0.071 mg. of potassium. Thus if 2 c.c. of 0.02 N potassium permanganate are originally added and 0.43 c.c. of the same solution is used in the final titration and 2 c.c. of 0.01 N sodium oxalate are required to decolorize the sample after the first oxidation, then $2.43 - 0.03$ (the blank) $\times 2$ (to change 0.02 N to 0.01 N) $- 2.00$ (c.c. of 0.01 N sodium oxalate added to decolorize the sample) $\times 7.1 = 19.88$ mg. potassium in 100 c.c. of serum.

Normal is 18 to 21 mg. per 100 c.c. To convert to meq./L., refer to page 415.

TESTS FOR CHLORIDES

Chloride Chemistry

Changes in chlorides and sodium in the body fluids occur simultaneously and in the same direction except under certain conditions. Plasma contains twice as much chloride as the red blood cells. The high concentration of protein in the cells limits the amount of base available for combination with electrolytes and ion. The chloride in the blood is expressed in terms of chloride ion. This varies from 340 to 370 mg. per 100 c.c. of plasma. One meq. of chloride ion corresponds to 35.5 mg., or 58.5 mg. per cent sodium chloride. When blood stands, there occurs a shift of chloride from plasma to cells, so that all specimens for this type of analysis should be sent to the laboratory as soon as possible after withdrawal. Chloride falls slightly during periods of active gastric secretion, owing to reabsorption from the intestine. The addition of sodium chloride to the intake of food is followed by but slight changes in the blood; even complete withdrawal results in very little change.

Increase in chlorides in the blood is not to be expected in kidney disturbance because of extrarenal factors. While chloride may be increased in nephrosis, it bears no relationship to the occurrence of edema. Hyperchloremia may occur in acute glomerulonephritis, also in complete obstruction of the urinary tract. There is no relationship between hypertension and chronic nephritis. Increased chloride may occur in essential hypertension, in cardiac decompensation, and following parenteral injections of sodium chloride. It was observed as a classic symptom (increase of chlorides in the urine) in the crises of pneumonia, alluded to in all the older textbooks on medicine.

Decrease of chlorides is seen following changes of sodium balance and concentration in the serum. Exceptions occur such as is seen in patients with excessive loss of gastric juice from vomiting. Here there is loss of chloride in the form of hydrochloric acid.

There is a decrease in Addison's disease, in pyloric stenosis, and other forms of high intestinal obstruction; severe diarrheas; pancreatic, biliary, and jejunal fistulas; excessive perspiration; the uremia of advanced glomerulonephritis; in lobar pneumonia before the crisis; and in some cases of hypoparathyroidism. For further information, consult the section on electrolyte balance and pages 407 to 409.

Chlorides in Plasma, Urine, and Other Fluids

Bedside Method of Scribner¹

Principle.—

An acidified sample is titrated with mercuric nitrate in the presence of diphenylcarbazone. The mercuric nitrate reacts with the chloride in the sample to form soluble but un-ionized mercuric chloride. When all the chloride is used up, the excess mercuric ion gives a strong purple color with diphenylcarbazone.

Reagents.—

Standard Sodium Chloride Solution, 100 meq./L.—

Dissolve 5.85 gm. pure, dry sodium chloride in distilled water and dilute to 1,000 c.c. with distilled water.

Mercuric Nitrate.—

Weigh 17.13 gm. fresh mercuric nitrate $[\text{Hg}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}]$ in a small beaker.

Add 50 to 100 c.c. distilled water.

Add 1 c.c. concentrated nitric acid.

Stir until the mercuric nitrate is dissolved.

Dilute to 1,000 c.c. with distilled water.

For units other than meq./L., use Table 29.

TABLE 29.—COMPOSITION OF STANDARDS AND OF TITRATING SOLUTIONS WHEN UNITS OTHER THAN MILLIEQUIVALENTS PER LITER ARE DESIRED

UNITS DESIRED	STANDARD SODIUM CHLORIDE, GM. PER LITER	MERCURIC NITRATE GM. PER LITER	CONCENTRATED NITRIC ACID IN MERCURIC NITRATE, C.C.
Mg. NaCl per 100 c.c.	10.00	29.30	2
Mg. Cl ⁻ (chloride) per 100 c.c.	8.25	24.20	2

Indicator.—

Dissolve 400 mg. diphenylcarbazone (Eastman Kodak No. 4459) in 100 c.c. of 95% ethyl alcohol.

Store in a light-tight bottle in a refrigerator.

This indicator must be made fresh at least every six months.

Approximately N/10 Nitric Acid.—

Dilute 7 c.c. fresh concentrated nitric acid to 1,000 c.c. with distilled water.

Equipment.—

4 2-ounce standard medicine dropper bottles with one-piece molded rubber caps.*

2 1-c.c. tuberculin syringes. One is used to measure the mercury solution and must be carefully selected. Its plunger should stop exactly at the 0 mark when pushed all the way down, or inconvenient corrections will have to be made during titration.

*Made by Armstrong Cork Co., Lancaster, Pa.

¹Scribner, B. H.: Proc. Staff Meet., Mayo Clin. 25: 209, 1950; Lab. Digest 14: 5, 1950.

- 1 1-ounce standard medicine dropper bottle. It should have a two-piece plastic top.
- 1 test tube about 1 inch outside diameter and 4 inches long.
- 1 test tube 3 inches long and of diameter great enough to allow the 4-inch tube to slip inside.

Assembling the Set.†—

Make the 1-ounce bottle for indicator light-tight with tape and black paper. Remove the droppers and cut the bulbs away from the molded rubber caps of two of the 2-ounce bottles and slip the tuberculin syringes through the holes. Leave the dropper in the cap of the bottle that is to contain acid, but remove it from the cap of the bottle that is to contain standard salt solution.

Drop the test tube of smaller diameter into the larger tube.

Group the bottles around the test tubes, positioning them as shown in Fig. 122. Tape all together. Label the bottles. Be sure that the specially selected syringe for mercury is in the mercury bottle.

Keep a little water in the bottle marked "sample" to prevent drying and sticking of the sample syringe.

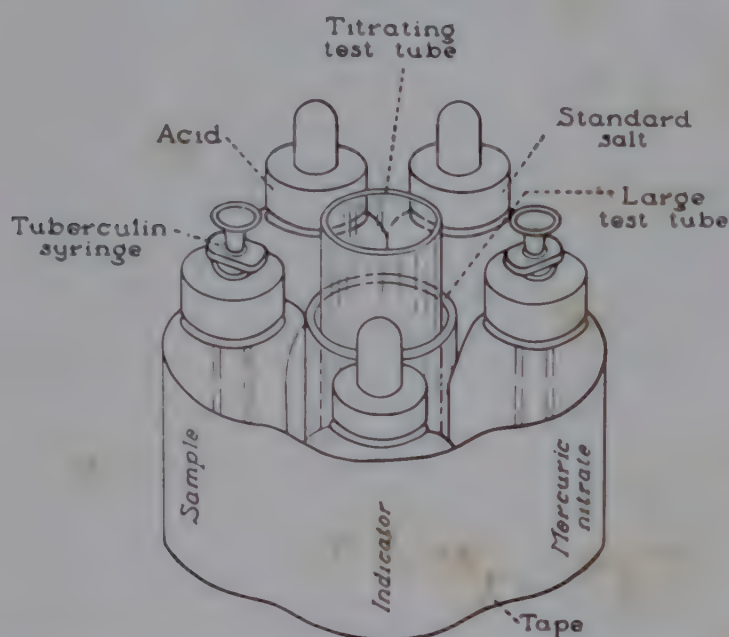


Fig. 122.—Set for bedside determination of chloride, method of Scribner. (Proc. Staff Meet., Mayo Clin. 85: 9, 1950.)

Renew the indicator each month from the supply kept in the refrigerator.

The test tube of larger diameter serves as a stand for the one of smaller diameter. The titration is performed in the smaller tube.

Blood Specimens.—

Do not expose the whole blood to the air unduly. Keep specimen in a stoppered container until the plasma or serum is separated from the cells. If time is not a factor, serum can be used after the clot has retracted. If the sedimentation rate of the patient is high, plasma can be obtained in a few minutes by allowing the blood to stand. Otherwise it must be centrifuged.

Measuring Solutions.—

Remove excess solution from outside of the syringe, when filling it, by touching the tip to the container holding that solution.

After delivering solutions into the titrating test tube, touch off the last drop on the side of the tube.

Rinse the syringe once with the sample to be tested before measuring the specimen for analysis. If sufficient sample is not obtainable for this step, the error still will probably not exceed $\pm 1\%$.

†Complete sets, including reagents, can be obtained from Rochester Products Co., Rochester, Minn.

After titrating, wipe the outside of the mercuric nitrate syringe free of sample to avoid diluting the reagent when the syringe is returned to the bottle.

Standardization of the Chloride Determination.—

Fill the sample syringe to the 1 c.c. mark with standard sodium chloride solution. Deliver this sample into the titrating test tube by pushing the plunger all the way down.

Complete the analysis for chloride as directed under "Technic."

Note the volume of mercury reagent required to react exactly with the standard sodium chloride solution.

Readjust the volume of the sodium chloride sample so that 1 c.c. of mercury reagent reacts exactly with it.

Example.—1.02 c.c. of mercury reagent is required to react exactly with the standard sodium chloride solution delivered from the 1 c.c. mark.

On the next try, the standard sodium chloride sample should be delivered from about the 0.98 c.c. mark.

Repeat this procedure until a plunger setting for the sample syringe is found such that the volume of standard sodium chloride solution delivered will react exactly with 1 c.c. of mercury reagent. Determine a new setting for each batch of mercury reagent or if new syringes are used. This setting is always used for measuring unknown samples.*

End Point.—

The purple end point with some specimens will not be as striking as with the standard sodium chloride solution. The first definite purple tint should be taken as the end point. This must not be confused with a salmon pink color that often precedes it. In hemolyzed plasma or plasma from jaundiced patients, the end point color is reddish brown instead of purple. In highly colored solutions such as bile or liquid stool, the end point must be taken as a darkening of the solution rather than a definite color change.

Technic.—

Fill the sample syringe to the correct setting with the specimen to be analyzed. Deliver the specimen into the titrating test tube.

Add 5 drops of indicator.

Add 3 dropperfuls (2 to 2.5 c.c.) of N/10 nitric acid.†

Fill the mercuric nitrate syringe to the 1 c.c. mark.

Place the syringe in the titrating test tube so that the molded rubber cap rests on the rim of the test tube.

Hold the test tube and cap with one hand, steadying the plunger with the index finger.

Add the mercuric nitrate slowly by pushing down the plunger with the other hand. Better control is obtained if the plunger is rotated as it is advanced.

Agitate the sample by shaking the test tube and syringe as a unit.

Add mercuric nitrate until the purple color appears, adding only a fraction of a drop at a time, as the end point is approached. A fraction of a drop may be added by washing it off the syringe tip with the sample.

Each 0.01 c.c. of mercuric nitrate solution used in the titration is equivalent to 1 meq. of chloride per liter, 10 mg. of chloride as NaCl per 100 c.c., or 5 mg. of chloride as Cl per 100 c.c., depending on how the mercuric nitrate solution was made (see Table 29).

Calculation.—

Read the result on the scale of the tuberculin syringe. The actual numbers on the scale must be reversed, since the zero point is at the distal end in the titration. Thus, 0.20 c.c. reading on the scale is actually 0.80 c.c. of reagent used.

*Standardization of the method and measurement of unknown samples are simplified if an attachment is used on the sample syringe to control the excursion of the plunger. Such an attachment is made by the Rochester Products Co., Rochester, Minn.

†A few specimens are so highly buffered with protein that 2 c.c. of N/10 acid does not lower the pH below 2. A premature and indistinct end point results. The addition of more acid will cause the end point color to fade. Six or more c.c. of N/10 acid should be added and the titration continued to the permanent end point.

Thus, if the syringe reads 0.20, 0.80 c.c. were used, and since each 0.01 c.c. corresponds to 1 meq./L. of chloride, this sample contains $0.80 \div 0.01$, or 80 meq./L.

Without changing the amount of indicator or N/10 nitric acid, the volume of the sample can be cut to a half or a fourth. The results are then doubled or quadrupled.

This method is adaptable to any liquid specimen, gastric juice, stool, urine, plasma, etc.

Blood Chlorides, Volhard-Arnold Method

Reagents.—

N/100 Acetic Acid.—

Add 0.6 c.c. glacial acetic acid to 1,000 c.c. distilled water. This solution must be made fresh each week.

Standard Silver Nitrate Solution.—(1 c.c. reacts with 0.001 gm. sodium chloride.)

Dissolve 2.906 gm. of silver nitrate in a small amount of distilled water in a liter volumetric flask.

Dilute to 1,000 c.c. with distilled water.

Keep in a brown bottle.

Standard Ammonium Thiocyanate Solution.—(1 c.c. reacts with 1 c.c. of the silver nitrate solution above, or 0.001 gm. sodium chloride.)

Dissolve 1.3 gm. of ammonium thiocyanate in about 800 c.c. distilled water.

Titrate against the above standard silver nitrate solution, using ferric alum indicator. Adjust by diluting with distilled water until 1 c.c. of this solution exactly equals 1 c.c. of the silver nitrate solution. The method of adjustment is described under normal solutions, pages 23 ff.

Ferric Alum Indicator.—

Dissolve 100 gm. of crystalline ferric ammonium sulphate, c.p., in 100 c.c. of 25% nitric acid (25 c.c. concentrated nitric acid to 100 c.c. total volume with distilled water).

Colloidal Iron.—

Colloidal iron is purchased from the dealer as dialyzed iron.

Equipment.—

2 50 c.c. centrifuge tubes graduated at 30 c.c.

1 15 c.c. volumetric pipette.

1 3 c.c. volumetric pipette.

1 4 inch funnel.

Filter paper.

2 10 c.c. volumetric pipettes.

1 25 c.c. volumetric flask.

1 1 c.c. graduated pipette.

1 20 c.c. volumetric pipette.

1 casserole.

1 buret.

Principle.—

Chloride is precipitated as silver chloride with silver nitrate, and the excess is titrated with thiocyanate, using ferric ammonium sulphate as an indicator.

Technic.—

Place in a 50 c.c. centrifuge tube, marked off at 30 c.c., 15 c.c. N/100 acetic acid.

Add 3 c.c. blood under the acid, washing out the pipette with the top fluid.

Mix until hemolysis is complete.

Dilute to 30 c.c. with distilled water and mix.

Place in a beaker of boiling water until coagulation is complete (usually about ten or fifteen minutes).

Stir occasionally.

Cool, and make up loss by evaporation.

Centrifuge for five minutes at 1,500 revolutions per minute.

Filter through filter paper, or pour off the supernatant fluid, into a large test tube.

This fluid may be slightly cloudy.

Add 6 drops of colloidal iron and mix.

Place the tube in a beaker of boiling water until flocculence occurs.

At this point, all the protein has been precipitated.

Filter through filter paper. The filtrate should be clear and colorless, and should not foam upon shaking.

Pipette 10 c.c. filtrate into a 25 c.c. volumetric flask.

Add 1 c.c. ferric alum indicator.

Add 10 c.c. of standard silver nitrate solution (1 c.c. equivalent to 0.001 gm. sodium chloride).

Dilute to 25 c.c. with distilled water.

Shake thoroughly for several minutes.

Transfer to a 50 c.c. centrifuge tube, and centrifuge for five minutes at 1,500 revolutions per minute.

Decant the clear supernatant fluid.

Pipette 20 c.c. supernatant fluid into a casserole.

Titrate with standard ammonium thiocyanate solution (1 c.c. equivalent to 1 c.c. standard silver nitrate solution, or 0.001 gm. sodium chloride).

The end point is a distinct faint yellow color, which is seen throughout the mixture.

Calculation.—

Divide the titration result (the number of c.c. thiocyanate used) by 0.8. Subtract the result from 10. This gives the amount of silver nitrate used in combining with the chlorides. Multiply by 0.001 to convert to grams of sodium chloride equivalent to the silver nitrate combined. Multiply by 100 to find the number of grams of sodium chloride in 100 c.c. blood, or the percentage.

$$\text{or } \left[10 - \left(\frac{\text{c.c. of thiocyanate used}}{0.8} \right) \right] \times 0.1 = \% \text{ of sodium chloride in 100 c.c. of blood.}$$

Example.—

The 0.8 represents the number of c.c. of blood used. (The 20 c.c. of supernatant fluid in the last step contains 0.8 c.c. of the original blood specimen.)

Suppose 3.2 c.c. of the thiocyanate solution were used in the titration.

3.2 divided by 0.8 is 4.

4 subtracted from 10 is 6, the amount of silver nitrate used in combining with the chlorides of the blood.

6 times 0.001 is 0.006 gm. of sodium chloride in 1 c.c. blood.

0.006 times 100 is 0.6 gm. of sodium chloride in 100 c.c. blood, or 0.6%.

Normal is 450 to 500 mg. or 0.45 to 0.5%.

1 meq. Cl. = 35.5 mg. or 58.5 mg. NaCl.

Blood Chlorides, Whitehorn¹

Reagents.—

Silver Nitrate Solution.—

Dissolve 4.791 gm. of silver nitrate in distilled water and dilute to 1 liter (1 c.c. is equivalent to 1 mg. of chlorine).

Potassium Thiocyanate Solution.—

Dissolve about 3 gm. of potassium thiocyanate in a liter of water. Standardize this solution against the silver nitrate solution by using 10 c.c. of the silver nitrate solution with 10 c.c. of water and ferric ammonium sulphate as indicator and titrating with the thiocyanate solution; 10 c.c. of the latter solution should be equivalent to 10 c.c. of the first solution.

¹J. Biol. Chem. 45: 449, 1921.

Powdered Ferric Ammonium Sulphate.—

Grind in a mortar a quantity of the ferric ammonium sulphate and store in a stoppered bottle.

Concentrated Nitric Acid.—**Equipment.—**

- 1 casserole.
- 1 10 c.c. volumetric pipette.
- 1 5 c.c. volumetric pipette.
- 1 25 c.c. or 100 c.c. cylinder graduated in 1.0 c.c.
- 1 buret.

Technic.—

Pipette 10 c.c. Folin protein-free filtrate (page 224) into a porcelain dish.

Add 5 c.c. standard silver nitrate solution.

Stir.

Add from a cylinder 5 c.c. of concentrated nitric acid.

Mix and allow to stand 5 minutes.

Add approximately 0.3 gm. of ferric ammonium sulphate.

Titrate the excess of silver nitrate with the standard thiocyanate solution until the definite salmon-red color persists for 15 seconds notwithstanding constant stirring.

Calculation.—

$(5 - \text{c.c. of thiocyanate used}) \times 100 = \text{mg. of chlorine in 100 c.c. blood.}$

To convert chlorine to sodium chloride, multiply the number of milligrams of chlorine by 1.65.

Example.—

1.1 c.c. of thiocyanate used.

$5 - 1.1 = 3.9 \times 100 = 390 \text{ mg. chlorine.}$

$390 \times 1.65^* = 643.5 \text{ mg. of sodium chloride in 100 c.c. blood.}$

Normal is 450 to 500 mg. sodium chloride in 100 c.c. blood.

1 meq. Cl. = 35.5 mg. or 58.5 mg. NaCl.

Blood Chlorides**Modified Method of Schales and Schales^{1, 2}****Reagents.—****Standard Sodium Chloride Solution.—**

Dry chemically pure sodium chloride at 120° C. to a constant weight.

Dissolve 0.5 gm. of this sodium chloride in 200 to 300 c.c. of distilled water in a liter volumetric flask and dilute to 1,000 c.c. with distilled water.

Mercuric Nitrate Solution.—

Dissolve 1.5 gm. mercuric nitrate, c.p.,

in 300 c.c. distilled water with the addition of 20 c.c. of 2 N nitric acid.

(Baker's analyzed mercuric nitrate is satisfactory.)

Dilute to 1,000 c.c. with distilled water.

Titrate against 5 c.c. of the standard sodium chloride solution, using 4 drops of the indicator, and adjust to exactly the same strength as the sodium chloride solution.

Indicator Solution.—

Dissolve 100 mg. of s-diphenylcarbazone

in 100 c.c. of 95% ethyl alcohol, and store in a dark bottle in the refrigerator. A fresh solution should be prepared each month.

*1.65 is the factor to convert chlorine to sodium chloride.

¹Modified by Division of Chemistry and Physics, Army Medical Center, Washington, D. C.

²Schales, Otto, and Schales Selma S.: J. Biol. Chem. 140: 879, 1941.

Technic.—

Pipette 5 c.c. of Folin-Wu protein-free blood filtrate into a porcelain dish.

Add 4 drops of indicator solution and mix.

Titrate with mercuric nitrate solution using a microburet calibrated in 0.01 c.c. Titrate to an intense violet-blue color which does not fade. Read from the burette the amount of mercuric nitrate solution used and calculate.

Calculation.—

C.c. of mercuric nitrate used $\times 100 =$ Mg. of chlorides as sodium chloride in 100 c.c. of blood. 1 meq. Cl. = 35.5 mg., or 58.5 mg. NaCl.

Note.—Baker's Analyzed mercuric nitrate, c.p., is a satisfactory grade. S-diphenyl-carbazone may be secured from the Eastman Kodak Co.

CHLORIDE DETERMINATION IN BLOOD SERUM OR PLASMA VERSUS DETERMINATION OF CHLORIDE IN RED BLOOD CELLS

The concentration of chloride in red blood cells is only about half that of the plasma or serum. Therefore, the concentration of chloride in whole blood is somewhat dependent upon the amount of red blood cell volume and does not indicate accurately the concentration in either plasma or serum. It would seem, therefore, that the determination of chloride in serum is to be preferred for clinical purposes. The methods outlined for the determination of chloride use the Folin-Wu filtrate.

Procedure Made Directly on Blood Serum.—

Deproteinization of blood serum is not necessary.

Dilute the serum sample, 0.5 c.c., with 4.5 c.c. of distilled water.

Carry out the determination as given for the filtrate, method of Schales and Schales.

The solution becomes light yellow before the end point changes and turns to pale violet at the end point.

Schales and Schales obtained results by direct titration that were slightly higher than those observed with filtrates.

If only small amounts of serum are available, the determination can be made by diluting 0.2 c.c. of serum with 1.8 c.c. of distilled water for the titration. The calculation is the same as for 2 c.c. of tungstic acid filtrate.

LaMotte Blood Chlorides Outfit

This set is used for the determination of chlorides, according to the method of Whitehorn. The chlorides are precipitated from the blood filtrate by means of silver nitrate in the presence of nitric acid, and the excess of silver titrated with standard thiocyanate solution, using ferric ammonium sulphate as an indicator. A special end point color standard is supplied to aid in detecting the end point of the titration.

TO MAKE A WATER-CHLORIDE BALANCE SHEET*

Save all urine liquid stool, gastric fluid, urine, etc., in marked containers. Add a dropperful of formaldehyde solution to each container to eliminate odor and to help prevent changes in pH. To insure that all specimens are saved, affix with tape, over the patient's toilet, a large sign reading "Save all specimens in designated containers." Place the sign in such a way that it must be moved before any specimen can be discarded.

Measure the specimens and analyze daily to determine total volume, chloride, and pH. Note total intake of water and chloride.

From these data construct a daily balance sheet as suggested in Table 30.

Application of Balance Sheet.—A patient may retain several hundred milliequivalents of sodium and chloride without developing edema. The

*Scribner, B. H.: Proc. Staff Meet., Mayo Clin. 25: 209, 1950.

balance sheet gives early warning of overdosage with sodium chloride long before edema actually develops. The warning is in the form of a markedly positive chloride balance. A negative chloride balance affords similar pro-

TABLE 30

A WATER-CHLORIDE BALANCE SHEET

From: 10 a.m. 11/21/49 To: 8 a.m. 11/22/49 Equals: 22 hours

INTAKE*			OUTPUT			
Volume (ml.)	Type of Fluid	Total Chloride (mEq.)	Volume (ml.)	Type of Fluid	Conc. of Chloride (mEq./L.)	Total Chloride (mEq.)
3,000	5 per cent dextrose in H ₂ O, parenteral	0	1,200	Urine	34	41
3,000	5 per cent dextrose in 0.9 per cent NaCl, parenteral	46.2	1,000	Insensible and sensible loss†	—	0‡
			2,100	Gastric aspirate	57	120
200	Oral fluid	0	1,700	Ileac content	114	194
6,200	Totals	46.2	6,000	Totals	—	355

*The water of oxidation is neglected; it probably averages around 250 ml. per 24 hours.

**pH is determined by the use of indicator paper. The paper used for this purpose is "Accutint Wide Range B" made by Analytischia Ltd., Montreal, Canada. However, a rough estimate of the pH can be obtained by noting the color of the sample when diphenylcarbazone is added. The sample will turn yellow when the pH is below 5; orange when the pH is between 5 and 7; and cherry red when the pH is above 8.

†The following guide has been found helpful in obtaining a rough estimate of 24-hour sensible and insensible loss: No fever and no perspiration—1,000 ml.; high fever or moderate perspiration—1,500 ml.; perspiration enough to require change of bed clothes—2,000 ml.; higher volumes for excessive perspiration.

‡Negligible chloride is lost with insensible perspiration. One can estimate that 70 meq. of chloride is lost with every 1,000 ml. of perspiration in excess of 1,000 ml. Thus if the loss was estimated at 3,000 ml. the chloride loss would be estimated at 140 meq.

From Scribner, B. H.: Proc. Staff Meet., Mayo Clin. 25: 209, 1950.

tection against a sodium chloride deficit long before it becomes detectable clinically. If desired, the actual changes in the volume of the extracellular

space can be calculated from the chloride balance.¹ The water-chloride balance sheet provides daily determinations of urine chlorides which in themselves can give useful information regarding electrolyte balance.

Disturbances of acid-base balance often can be anticipated by noting the pH of the various specimens; e.g., if a large volume of gastric juice is lost and its pH is 1.5, alkalosis might be suspected.

The most useful application is its use in planning administration of fluids from day to day. By noting the figures representing the output of water and chloride for the day preceding, one can estimate the output for the next day and plan treatment accordingly.

TESTS FOR CALCIUM

There is a close relationship between the calcium and phosphorus in the blood. There is very little calcium in the blood cells. **Normally**, the serum or plasma shows between 9.3 and 10.3 mg. in 100 c.c. for adults and between 10 to 11 for children. Usually, 10 mg. are considered the concentration in health in a given individual. Fifty per cent of the 10 mg. is diffusible, and the remainder is loosely combined with the serum proteins. Phosphates and carbonates form insoluble calcium salts; fatty acids prevent the absorption of calcium by forming calcium soaps. If the amount of fat in the diet is increased, the amount of calcium bound to fatty acid in the stools and the amount of phosphates in the urine are increased. If bile is excluded from the gastrointestinal tract, thus interfering with the absorption of fats, this increases the ratio of urine phosphate to fecal phosphate and increases the amount of calcium soap in the stools, actually causing a decrease of serum calcium. About one-third of the freely diffusible calcium is physiologically active. The concentration of the freely diffusible calcium is greater than its solubility; therefore, fractions other than the serum proteins must be concerned with keeping the calcium in solution.

There seems to be great difficulty in increasing the amount of blood calcium by the ingestion of food or injection of calcium salts even in large amounts. Sunlight affects the calcium concentration of the serum; hence the seasonal variation in serum calcium which has been frequently noted. These effects are due to ultraviolet rays. Cod-liver oil seems to have a regulatory effect on calcium and phosphorus metabolism. Temporary starvation has little effect upon the concentration of calcium in the serum; but in chronic malnutrition, the reduction in serum calcium is proportional to the serum protein deficiency.

Calcium has numerous functions in the human body, not only as a structural factor in the bones and teeth, but also as a prerequisite to normal neuromuscular action and the clotting of blood. It seems to exert a significant influence on the movement and distribution of other mineral elements in the body. Milk seems to be the best source of calcium, although vegetables also contribute this element to the diet. Animals seem to obtain their calcium requirement more easily from the ingestion of milk than from the ingestion of

¹Elkinton and Winkler: J. Clin. Invest. 23: 93, 1944.

vegetables. In a report made by Shields¹ and his co-workers, it was shown that if the availability of calcium in milk is expressed as 100, carrots, fresh lettuce, and string beans have the respective values of 85, 80, and 74 in this connection. They concluded that there is something in vegetables which decreased the utilization of the contained calcium over that in liquid or dried milk. Another important item about the availability of calcium from different foods is the efficiency with which the body can utilize the calcium in an excellent source such as milk. Studies from the University of Illinois² made on this question in preschool children and in adult human subjects are interesting. In these experiments the conception of degree of utilization was the relationship between the net calcium loss to the body on a calcium-poor basal ration and the supplementary dietary calcium required to eliminate that net loss. This method of computation when applied to dried or fluid milk showed that only 20 per cent of the calcium in this relatively high calcium food was utilized under the conditions of study by the children and the adults.

Regarding the calcium requirement of man, there are two reports³ published on studies of a total of sixteen adults in which milk provided a large part of the calcium. These studies showed essential agreement in daily calcium requirement, the value being 9.6 mg. per kilogram of body weight from one study and 10.7 mg. from the other. As intimated above, temporary shortage of dietary calcium is not of immediate concern because of the considerable reserves of calcium in the body. The newer studies cited, however, show the value in long range planning of dietaries respecting the usefulness and availability of calcium.

The metabolism of calcium is closely connected with the parathyroid gland function. Parathyroid hormone injections cause an immediate increase in the urinary excretion of phosphorus with consequent lowering of the serum phosphorus. As stated above, there is a close relationship between phosphorus and calcium, so that with the lowering of the serum phosphorus there is an increase in serum calcium. This is followed by increased calcium excretion, mostly to be noted in the urine.

In natural hyperfunction of the parathyroids, or in parathyroid adenoma, serum calcium may or may not be increased unless there is severe renal damage with retention, in which case it may rise as high as 23.6 mg. per hundred cubic centimeters; serum phosphorus falls from a normal level of 4 to 5 mg. per hundred cubic centimeters to as low as 1.4; and urinary calcium is increased.

Decreased calcium metabolism, with lowering of the serum calcium, and tetany are closely associated. Tetany may be differentiated into the following types: (1) gastric tetany; (2) tetany of hyperventilation; (3) infantile tetany; (4) tetany of pregnancy or lactation; and (5) tetany following accidental extirpation of parathyroid tissue.

¹Shields, J. B., Fairbanks, B. W., Berryman, G. H., and Mitchell, H. H.: *J. Nutrition* 20: 263, 1940.

²Steggerda, F. R., and Mitchell, H. H.: *J. Nutrition* 17: 253, 1939; 21: 577, 1941. Kinsman, Gladys, Sheldon, Dorothy, Jensen, Elizabeth, Bernds, Marie, Outhouse, Julia, and Mitchell, H. H.: *ibid.* 17: 429, 1939. Breiter, Herta, Mills, Rosaline, Dwight, Julia, McKey, Beula, Armstrong, Williamina, and Outhouse, Julia: *ibid.* 21: 351, 1941.

³Steggerda, F. R., and Mitchell, H. H.: *J. Nutrition* 21: 577, 1941. Outhouse, Julia, Breiter, Herta, Rutherford, Esther, Dwight, Julia, Mills, Rosaline, and Armstrong, Williamina: *ibid.* 21: 565, 1941.

Calcium determinations are important in establishing hyper- or hypofunction of the parathyroid glands.

Calcium and Magnesium in Serum

Method of Coulson and Hernandez

Enders,¹ in 1938, synthesized the sodium salt of ethylenediaminetetraacetic acid (EDTA). This compound forms soluble nonionized complexes with calcium and magnesium and prevents the formation of insoluble soaps. It has been adopted by many industrial concerns for water purification. Schwarzenbach² devised methods of estimating calcium and magnesium by titration with EDTA, using the azo dye Eriochrome Black T as an indicator.

Principle.—

Ethylenediaminetetraacetic acid (trade names Versene and Sequestrene) forms highly soluble but weakly ionized salts with calcium and magnesium. An indicator, "Eriochrome Black T," turns red in the presence of calcium or magnesium and blue-green in their absence. Serum or heparinized plasma is titrated directly with standardized EDTA to a blue-green color, and the total milliequivalents of calcium plus magnesium are computed. From the total titer in meq./L., the figure 1.64, representing the average human plasma magnesium in meq./L., is subtracted, the remainder being calcium.

Reagents.—

EDTA Standard (Ethylenediaminetetraacetic Acid).—

Dissolve 0.5 gm. of pure dry Na_2EDTA^3
in 1,000 c.c. of distilled water.

Eriochrome Black T Indicator.—

Place about 5 gm. of Eriochrome Black T dry buffer mixture⁴ in a 100 c.c. volumetric flask.

Add 4 c.c. of concentrated ammonium hydroxide.

Dilute to 100 c.c. with distilled water.

Shake to dissolve as much as possible.

Keep cold and dark when not in use.

This indicator should be made fresh daily. It may be kept frozen without deterioration for several weeks.

Buffer Solution.—

Dilute 1 c.c. of ethanolamine (cholamine) to 100 c.c. with distilled water.

Technic.—

Standardization of EDTA.—

Fill a semimicroburette with standard EDTA.

Place 5 c.c. of buffer solution and 1 c.c. of indicator in a 50 c.c. beaker.

Add the EDTA *very carefully* to the buffer-indicator mixture until the last trace of red in the mixture disappears. Pure ingredients would not require the addition of EDTA since they would be calcium-free.

Add exactly 1 mg. of calcium (1 c.c. of a 10 mg. % standard solution).

Titrate to a blue-green end point.

Calculate the concentration of the standard in terms of milliequivalents. 20 mg. of Ca /liter = 1 meq./L.

¹Enders: *Fette u. Seifen*. 45: 144, 1938.

²Schwarzenbach and Ackerman, *Helvet. chem. acta* 30: 1789, 1949. Schwarzenbach and Biederman, *Helvet. chem. acta* 31: 459, 1948. Schwarzenbach and Ackerman, *Helvet. chem. acta* 31: 1029, 1948.

³EDTA is available from Bersworth Chemical Co., Framingham, Mass.

⁴Univ.-II Curtin Co., Cat. No. 38726V.

Determination of Serum Calcium.—

Place 5 c.c. of buffer solution and 1 c.c. of indicator in a 50 c.c. beaker.

Remove any trace of red with EDTA as before.

Add 1 c.c. of serum to the beaker.

Titrate with EDTA to a blue-green end point.

Less than 1.0 c.c. of plasma may be used if the usual precautions in microanalysis are observed. *

Calculations.—

If the standard EDTA is titrated against 0.1 mg. of calcium (0.005 milliequivalent), and 1 c.c. of serum is used, the following equation may be used.

$$\frac{\text{Vol. EDTA used to titrate}}{0.1 \text{ mg. Ca}} = \frac{5}{\text{X milliequivalents of calcium + magnesium per liter for each c.c. of standard EDTA used in titrating the serum.}}$$

X times the c.c. needed to titrate the 1 c.c. of serum = calcium + magnesium in meq./L. To calculate calcium alone, subtract 1.64 and the answer will be in milliequivalents of calcium per liter. To convert calcium in meq./L. to mg. %, multiply by 2.

From all available evidence at present, the plasma magnesium level is subject to little variation. Clinical tests of this method of calcium determination have proved quite satisfactory. For the most accurate method of magnesium analysis, determine the calcium by the usual KMnO_4 method and subtract this value from the total magnesium + calcium as determined by EDTA titration.

Calcium, Clark Method***Reagents.—****Saturated Solution of Sodium Citrate, pH 7.4.—**

Dissolve 92 gm. sodium citrate, c. p., in 100 c.c. distilled water with heat.

Filter when cool.

Adjust the reaction to pH 7.4. (See culture media, page 1422.)

3% Ammonium Oxalate Solution.—

Dissolve 3 gm. of ammonium oxalate, c. p., in 50 c.c. distilled water in a 100 c.c. volumetric flask.

Dilute to 100 c.c. with distilled water.

Approximately Normal Sulphuric Acid.—

To 800 c.c. distilled water add 28 c.c. concentrated sulphuric acid, c. p.

Dilute to 1,000 c.c. with distilled water.

N/10 Potassium Permanganate.—

Dissolve 3.161 gm. of potassium permanganate in enough distilled water to make a total of 1,000 c.c.

Be sure all the permanganate is in solution, then filter through glass wool. Titrate against a N/10 oxalic acid solution to which has been added (in the casserole) about 5 c.c. of approximately normal sulphuric acid. The end point is reached when a faint permanent pink is obtained. Keep the solutions in the casserole warm.

N/10 Oxalic Acid.—

Dissolve 6.3035 gm. of oxalic acid ($\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$) in

500 c.c. distilled water in a liter volumetric flask, and dilute to 1,000 c.c. with distilled water.

The method of titration of potassium permanganate solutions against oxalic acid solutions is given on page 24.

N/100 Potassium Permanganate.—(1 c.c. is equivalent to 0.2 mg. calcium.)

Add 10 c.c. N/10 potassium permanganate to 50 c.c. distilled water in a 100 c.c. volumetric flask.

Dilute to 100 c.c. with distilled water.

*J. Biol. Chem. 63: 461, 1925.

Since calcium is essentially a constituent of the plasma, and not the cells, the technic will be outlined for serum and plasma. Obviously, if plasma is to be used, it must be *citrated*, not *oxalated*. One c.c. sodium citrate solution (containing about 900 mg. sodium citrate, pH 7.4) is adequate for 10 c.c. of blood, although this is about the minimum quantity. Withdraw 10 c.c. of blood from the vein, in syringe containing 1 c.c. of sodium citrate (containing 900 mg. to each c.c.) centrifuge and use plasma. Or take the blood as for a Wassermann test (Chapter XIII), allow it to clot, centrifuge fifteen minutes, and use the serum. Serum is preferable to plasma for the calcium estimation.

Principle.—

Calcium is precipitated from the serum or plasma as calcium oxalate. The calcium oxalate is then removed by centrifugalization. Upon the addition of sulphuric acid to the precipitate, oxalic acid is liberated which is titrated using a standard solution of potassium permanganate which oxidizes the oxalic acid.

Technic.—

In a 50 c.c. centrifuge tube place 1 to 5 c.c. of serum or citrated plasma.

While rotating the tube, add slowly 3% ammonium oxalate, equal in volume to one-half the amount of serum or plasma used.

Mix thoroughly, and allow to stand overnight.

Rub down the walls of the tube with a rubber policeman (washing the policeman with a small amount of distilled water).

Centrifuge at 1,500 revolutions per minute for five minutes.

Remove the supernatant liquid completely by a siphon or by careful decantation.

Stir up the precipitate with a fine stream of cold distilled water, washing down the walls of the tube, using in all approximately 35 c.c. of water.

Centrifuge immediately and completely siphon off the wash water.*

Dissolve the precipitate in 5 c.c. of approximately normal sulphuric acid heated to 75° C.

Titrate, warm (75° C.), against N/100 potassium permanganate to the first faint permanent pink.

Calculation.—

$$\text{No. c.c. potassium permanganate used} \times 0.2 \times \frac{100}{\text{c.c. of serum used}} = \text{mg. calcium in 100 c.c. of serum.}$$

Example.—

5 c.c. of serum were used in the test.

2.6 c.c. of N/100 potassium permanganate were used in titration.

$$2.6 \times 0.2 \times \frac{100}{5} = 10.4 \text{ mg. calcium in 100 c.c. of serum.}$$

Normal is 9 to 11 mg. in 100 c.c. serum.

Calcium, Clark-Collip Method^{1, 2}

Principle.—

The calcium of blood serum is precipitated as calcium oxalate and the amount determined by titration with a standard permanganate solution.

Reagents.—

4% Ammonium Oxalate.—

Dissolve 4 gm. ammonium oxalate in water and dilute to 100 c.c.

Reliable 6 months.

*It is extremely important that all of the excess ammonium oxalate be thoroughly washed out.

¹Clark, G. W.: J. Biol. Chem. 49: 487, 1921.

²Clark, G. W., and Collip, J. B.: J. Biol. Chem. 63: 461, 1925.

Sulphuric Acid, Approximately N/1.—

Dilute 28 c.c. of concentrated sulphuric acid to 1 liter.

Reliable 1 year.

N/100 Potassium Permanganate.—

Dilute 10 c.c. of N/10 potassium permanganate to 100 c.c. This solution is not sufficiently accurate for use and also changes its strength on standing. It should be titrated each time an analysis for calcium is made.

N/100 Sodium Oxalate.—

To a 100 c.c. volumetric flask add exactly 10 c.c. of N/10 sodium oxalate solution. Dilute to the mark with normal sulphuric acid solution. Mix.

Reliable for 6 months.

2% Ammonium Hydroxide.—

Dilute 2 c.c. of ammonium hydroxide to 100 c.c.

Reliable for 6 months.

Technic.—

In a tube place 8 to 10 c.c. of blood. Allow it to clot, and separate serum by centrifuging.

Into a centrifuge tube pipette 2 c.c. water, 2 c.c. serum, and 1 c.c. of 4% ammonium oxalate solution.

Mix thoroughly, without stoppering, and allow specimen to stand 30 minutes.

Centrifuge at 1,500 r.p.m. for 5 minutes.

Carefully pour off supernatant liquid.

While the tube is still inverted, place it in a rack for 2 minutes to drain, the mouth of the tube resting on filter paper.

Wipe the mouth of the tube dry.

Stir the precipitate and wash down the sides of the tube with about 5 c.c. of 2% ammonium hydroxide solution.

Centrifuge and drain as before.

Repeat the treatment with ammonium hydroxide solution and centrifuging.

Blow 2 c.c. of the approximately N/1 sulphuric acid solution from a pipette directly upon the precipitate to break up the mat and facilitate solution.

Place the tube in boiling water for about 1 minute. Titrate at 70° to 75° C. with N/100 potassium permanganate solution to the first faint pink persisting for about 1 minute.

Place 2 c.c. of N/100 sodium oxalate solution in a centrifuge tube. Heat and titrate it with potassium permanganate heated at 70° to 75° C., persisting for about 15 seconds. Use this for calculating the factor.

Calculation.—

$T =$ c.c. potassium permanganate used in titration of unknown.

$F = \frac{2}{\text{c.c. potassium permanganate used in titration of N/100 sodium oxalate}}$

$X =$ mg. calcium in 100 c.c. of blood serum; or,

$X = T \times F \times 10$

Example.—

2 c.c. N/100 potassium permanganate used in titration of unknown.

4 c.c. N/100 potassium permanganate used in titration of N/100 sodium oxalate for factor.

$2 \times \frac{2}{4} \times 10 = 10$ mg. calcium in 100 c.c. of serum.

Normal.—9 to 11 mg. calcium in 100 c.c. of serum.

Calcium, Turbidimetric Technic

This technic was described by Barney and Sulkowitch¹ and is a method to estimate roughly the amount of calcium in urine and thus to calculate inferentially the blood level.

¹Barney, J. D., and Sulkowitch, H. W.: J. Urol. 37: 751, 1937.

Fennel² has used this method for the clinical determination of the calcium concentration in serum and a more accurate estimation of its concentration in urine.

Turbidimetric Serum Calcium Determination, Fennel

Sulkowitch's reagent.—

Dissolve 2.5 gm. oxalic acid.
 2.5 gm. ammonium oxalate.
 5.0 gm. glacial acetic acid in enough distilled water
to make 150.0 c.c.

Mix equal volumes of urine and Sulkowitch's reagent.

If there is no precipitation, the serum calcium may be presumed to range somewhere below 8.5 mg. %. If there is a fine white precipitate, the serum concentration probably lies between 8.5 and 10 mg. %. If there is a heavy milky precipitate, the serum concentration is probably above 12.5 mg. %, definitely in the pathologic range.

Technic.—

Use either a "to deliver" or a "to contain" pipette but always rigidly use the same technic.

Place in a test tube 1 c.c. of serum and
 add 4 c.c. of physiologic saline solution.

Place 2 c.c. of Sulkowitch reagent in another test tube.

Rapidly pour the Sulkowitch reagent into the serum-saline mixture, invert five or six times, stopper with a clean rubber stopper, and set aside for a given (unvarying) length of time. Thirty minutes is usually used. Invert several times during this period. This gives varying degrees of turbidity, depending on the concentration of the calcium. Other factors which may play a minor role do not appear to be of clinical importance.

At the same time prepare a color blank. Mix 1 c.c. of serum with 6 c.c. of saline solution. This is used to determine the "null point" on the colorimeter, before reading the unknown. Blue and green filters give almost identical readings. The green is preferable.

If the colorimeter is adapted for micromethods, use only half the above quantities, 1 c.c. of serum serving for both the blank and the unknown.

After the thirty-minute period, insert the color control in the colorimeter. Bring the milliammeter to the null point. Substitute the unknown, and read.

To calibrate the colorimeter for serum calcium.—

10% Calcium Chloride Stock Solution.—

Dissolve 10 gm. Merck's reagent grade calcium chloride, neutral crystals,
 $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, molecular weight 219.08,
in enough distilled water to make 100 c.c. solution.

0.05% Solution of Calcium Chloride.—

Dilute 1 c.c. of the stock 10% solution of calcium chloride to 200 c.c. with distilled water, using a volumetric flask.

One molecule of calcium, with a molecular weight of 40.08, makes up only 40.08/219.08 of each gram of calcium chloride. Thus, each 0.1 c.c. of the 0.05% solution represents a concentration of 0.915 mg. per 100 c.c.

To a series of five tubes, add, respectively 0, 0.1, 0.4, 0.6, and 0.8 c.c. of 0.05% solution of calcium chloride.

Bring the contents of each tube to a volume of 4 c.c. with physiologic saline solution.

Add to each tube 1.0 c.c. of pooled normal serum on which a chemical calcium determination has already been made (serum left from serologic tests may be used). Mix by inverting several times.

To each of five lipped test tubes, add 2 c.c. of Sulkowitch's reagent.

²Fennel, E. A.: Proc. Staff Meet. Clin., Honolulu 8: 98-99, 1942.

Typical Titration (Fennel)

TABLE 31.—SERUM CALCIUM CALIBRATION (GREEN FILTER)

TUBE NO.	1	2	3	4	5	6 (NULL CONTROL)
CaCl ₂ 0.05%						
0.1 c.c. = 0.915 mg. %	0.0	0.1	0.4	0.6	0.8	0.0
Saline	4.0	3.9	3.6	3.4	3.2	6.0
Pooled Serum at 10.1 mg. %	1.0	1.0	1.0	1.0	1.0	1.0
Sulkowitch Reagent	2.0	2.0	2.0	2.0	2.0	0.0
Calcium Concentration	10.1	11.015	13.760	15.590	17.420	
Colorimeter Reading	71	67	59	53	48	84; then adjust to null of 100

Pour the reagent rapidly from these into the serum-saline-calcium solutions. Mix the contents of the tubes rapidly by inversion and allow to stand for the standard time, thirty minutes.

Make a control for the null point by adding 6 c.c. of saline to 1 c.c. of serum. At the end of thirty minutes, use the control to set the photoelectric colorimeter to the null point. Read the dilutions of all the tubes in the apparatus.

The concentrations of the several tubes is equal to the chemically determined concentration of calcium in the pooled serum plus 0.915 mg. % for each 0.1 c.c. of 0.05% calcium chloride solution that has been added.

If the technic was careful, these points, plotted on graph paper, fall on a reasonably straight line.

Urine Calcium

An aliquot portion of a twenty-four-hour specimen *must* be used. To quote Fennel, "Anything but an aliquot portion of a twenty-four-hour specimen is a delusion." Calcium excretion rates are not equal at any two moments of the day. Use either a sample of a twenty-four-hour, well-mixed specimen, or better a series of twenty-four-hour specimens, for definite valuable clinical information. If the intake is low, a low output is to be expected, and vice versa. In order to interpret the results, the intake should be known with reasonable accuracy.

Turbidimetric Determination of Urine Calcium

- Place 1 c.c. of urine in a test tube.
- Add 4 c.c. distilled water.
- Mix thoroughly.
- Add suddenly 2 c.c. of Sulkowitch's reagent and mix thoroughly.
- Allow to stand for thirty minutes, inverting several times during the period.
- Read in the colorimeter, preferably with a green filter.

The average "amber" or even "dark amber" urine, diluted 1:7 in this way, is so nearly colorless that a dilute urine control for setting the null point is unnecessary. Distilled water may be used instead. If the urine is deeply pigmented or bile stained, a null point blank may be prepared easily by using 1 c.c. of urine and 6 c.c. of distilled water.

The colorimeter may be calibrated for urine calcium determinations as explained in Table 32.

TABLE 32.—URINE CALCIUM CALIBRATION (GREEN FILTER)

TUBE NO.	1	2	3	4	5	6	7
CaCl ₂ 0.05%	0.5	1.0	1.5	2.0	2.5	3.0	3.5
1.0 c.c. = 9.150 mg. %							
Water (c.c.)	4.5	4.0	3.5	3.0	2.5	2.0	1.5
Sulkowitch Reagent (c.c.)	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Concentration (mg. %)	4.575	9.150	13.925	18.300	22.875	27.450	53
Mg./1500 c.c.	67.5	137.2	208.5	274.5	343.0	412.0	52.025
Colorimeter Reading (est. 24 hours)	92.5	84	75	69	60	57	480.0

Calcium in Blood Serum, Method of Roe and Kahn

Reagents.—

10% Trichloroacetic Acid Solution, Molybdic Acid Reagent, and Sulphonic Acid Reagent.—

These are the same as used in the determination of inorganic phosphorus, method of Fiske and SubbaRow, page 446.

Stock Calcium Solution.—

Dissolve 0.4991 gm. of pure calcium carbonate (Iceland spar)
in 50 c.c. of 10% trichloroacetic acid in a liter volumetric flask.

Shake well, and when evolution of CO_2 has ceased, dilute to 1000 c.c. with 10% trichloroacetic acid.

Standard Calcium Solution.—

Place 10 c.c. of stock calcium solution in a 100 c.c. volumetric flask.

Add 70 c.c. of 10% trichloroacetic acid.

Dilute to 100 c.c. with distilled water.

5 c.c. = 0.1 mg. of calcium.

Alkaline Alcohol Wash Reagent.—

Place 58 c.c. of 95% ethyl alcohol in a 100 c.c. graduated cylinder.

Add 10 c.c. of amyl alcohol.

Dilute to 100 c.c. with distilled water.

Add 2 drops of 1% phenolphthalein.

Add 5% sodium hydroxide, a drop at a time, with repeated shaking, until a distinct pink color is obtained.

25% Sodium Hydroxide Solution.—

Dissolve 25 gm. of sodium hydroxide, c.p., in enough distilled water to make 100 c.c. solution.

5% Trisodium Phosphate Solution.—

Dissolve 5 gm. trisodium phosphate (Na_3PO_4) in enough distilled water to make 100 c.c. solution.

Technic.—

Prepare a filtrate using 10% trichloroacetic acid in the manner described under inorganic phosphorus, page 450.

Use graduated centrifuge tubes the tips of which are sufficiently narrow so that the diameter at the 0.1 c.c. mark does not exceed 7 mm. The tips must not be too finely drawn out. Tubes must be absolutely clean. When not in use, keep them immersed in the dichromate-sulphuric acid cleaning solution. Since inaccuracies in graduation sometimes occur in these tubes, be sure to check the graduations, and especially the 10 c.c. mark, and discard or re-graduate those tubes found to be inaccurate.

Into a graduated centrifuge tube marked "U" for unknown, place 5 c.c. of the filtrate.

Place 5 c.c. of the standard calcium solution containing 0.1 mg. of calcium in a similar tube marked "S" for standard.

Add to both tubes 1 c.c. of 25% sodium hydroxide solution, mix, and allow to stand for 5 minutes.

Add to both tubes 1 c.c. of the trisodium phosphate solution and allow to stand for 1 hour.

Centrifuge for 2 minutes, decant the supernatant fluid **but avoid disturbing the precipitate.**

With the mouth of the tube inverted, allow the tube to drain for several minutes, after which wipe out any remaining fluid from within the mouth of the tube with a piece of filter paper.

Blow forcefully from a pipette about 3 c.c. of the alkaline alcohol wash so as to break up the mat of precipitate. If the precipitate is not broken up in this manner, it must be disturbed with a glass stirring rod.

Wash down the walls of the tube and the stirring rod with an additional 2 c.c. of the alkaline alcohol wash.

Centrifuge the tubes for 2 minutes, decant the fluid, and drain as indicated above.

Redissolve the precipitate in both the standard and the unknown with 4 c.c. of 10% trichloroacetic acid reagent.

Add to each tube 1 c.c. of molybdic acid reagent and 0.4 c.c. of the sulphonic acid reagent.

Dilute to 10 c.c. with distilled water, mix, and allow to stand for 10 minutes.

Compare in the colorimeter.

Calculations.—

$$\frac{\text{Reading of the Standard}}{\text{Reading of the Unknown}} \times 10 = \text{Mg. of calcium per 100 c.c. of blood.}$$

Note.—Use the trichloroacetic acid filtrate to send a specimen to a distant laboratory for determination.

Normal.—9 to 11 mg. per 100 c.c. of serum.

LaMotte Blood Calcium-Phosphorus Outfit*

This is a set for determining the amounts of calcium and phosphorus in the serum and their exact ratio to each other. The standard procedure has also been adapted to a micro procedure which is valuable when working with children. Both calcium and phosphorus are determined in the same deproteinized filtrate. The complete calcium determination is made in a single tube, thereby reducing the chance of error to a minimum. The micro test requires only 0.2 c.c. of serum for the calcium determination and 0.1 c.c. for phosphorus.

INORGANIC PHOSPHORUS

Kuttner and Lichtenstein Method

Reagents.—

7 per cent Trichloroacetic Acid.—

Dissolve 7 gm. trichloroacetic acid in enough distilled water to make 100 c.c. of solution.

10 N Sulphuric Acid.—

Add 280.35 c.c. of sulphuric acid, c.p., sp. gr. 1.84 to about 500 c.c. of distilled water in a liter volumetric flask. Add slowly and cool under running water while adding.

Dilute to 1000 c.c. with distilled water, after cooling.

7.5 Per Cent Sodium Molybdate Solution.—

Dissolve 7.5 gm. sodium molybdate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) in enough distilled water to make 100 c.c.

Stannous Chloride Stock Solution.—

Dissolve 10 grams of stannous chloride (SnCl_2) (or 11.9 gm. of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$) in 25 c.c. concentrated hydrochloric acid.

Store in a brown glass-stoppered bottle. Before use, prepare a 1:200 dilution by adding 0.1 c.c. to enough distilled water to make 20 c.c.

Standard Phosphate Stock Solution (1 c.c. equals 0.1 mg. P).—

Place 0.4394 gm. monobasic potassium phosphate (KH_2PO_4) in a liter volumetric flask.

Dissolve in distilled water and dilute to 1 liter.

Add a few drops of chloroform to prevent growth of molds.

Dilute Standard Phosphate Solution (1 c.c. equals 0.01 mg. P).—

Dilute 1 c.c. of the standard phosphate stock solution with 9 c.c. distilled water.

*Roe and Kahn: J. Biol. Chem. 67: 3, 1926.

Principle.—

The trichloroacetic acid filtrate is treated with sulphuric acid and sodium molybdate which forms phosphomolybdate. The phosphomolybdate is reduced by the stannous chloride which forms a blue color due to colloidal reduced oxides of molybdenum. A standard solution of phosphorus is treated in a similar manner and the unknown is compared colorimetrically with it.

Technic.—**Preparation of Unknown.—**

Place 9 c.c. of 7% trichloroacetic acid in a tube.

Add 1 c.c. serum.

Shake and let stand for 10 minutes.

Filter through phosphorus-free filter paper.

Transfer 5 c.c. supernatant fluid to another tube.

Add 1 c.c. 10 N sulphuric acid
and 2 c.c. distilled water.

Shake.

Add 1 c.c. sodium molybdate solution and mix.

Add 1 c.c. dilute stannous chloride solution and mix.

Compare within two hours with standards prepared simultaneously.

Preparation of Standard.—

Unless a correction factor is used, the phosphorus content of the unknown should not differ more than one-third from the standard solution.

Use 1.0, 1.5, 2.0, or 3.0 c.c. of the standard solution.

Add 1 c.c. 10 N sulphuric acid
and enough distilled water to make up to 8 c.c.

Add 1 c.c. of 7.5 per cent sodium molybdate solution and mix.

Add 1 c.c. dilute stannous chloride and mix.

Within two hours, compare in colorimeter, with the standard set at 15 mm.

Calculation.—

$$\frac{\text{Standard 15 mm.}}{\text{Reading of Unknown}} \times \text{mg. P in standard} \times \frac{100}{0.5} = \text{mg. P in 100 c.c.}$$

Normal phosphorus for adults is 3.7 mg. per 100 c.c.; for children, 5 mg. phosphorus per 100 c.c.

Benedict Method**Reagents.—****20% Trichloroacetic Acid.—**

Dissolve 20 grams of trichloroacetic acid, c. p., in 50 c.c. distilled water, and dilute to 100 c.c. with distilled water.

Standard Phosphate Solution.—(3 c.c. contain 0.025 mg. of phosphorus.)

Dissolve 0.0351 gram of monobasic potassium phosphate, c. p. (KH_2PO_4), in 500 c.c. of distilled water in a liter volumetric flask.

Dilute to 1000 c.c. with distilled water.

Hydroquinone-Bisulphite Reagent, Benedict.—

Dissolve 30 grams of sodium bisulphite, c. p., and 1 gram of hydroquinone in about 100 c.c. of distilled water, and dilute to 200 c.c. with distilled water.

Molybdic Acid Reagent, Benedict.—

Dissolve 20 grams of molybdic acid (99.9%) in 25 c.c. of 20% sodium hydroxide solution with heat.

Dilute to 200 c.c. with distilled water.

Filter if necessary.

Add slowly, and with constant agitation under cold running water, 200 c.c. of concentrated sulphuric acid.

This reagent possesses a fairly deep blue color, but on dilution with the unknown or the standard, previous to heating, the color disappears.

Equipment.—

- 1 5 c.c. volumetric pipette.
- 2 3 c.c. volumetric pipettes.
- 3 2 c.c. volumetric pipettes.
- 2 1 c.c. graduated pipettes.
- 1 large test tube.
- 1 2 inch funnel.
- 1 small piece of dry, ashless filter paper.
- 2 sugar tubes.
- 1 beaker or cup of boiling water.

Principle.—

Protein is precipitated from the serum or plasma by trichloroacetic acid. After filtration, the filtrate contains the phosphorus in the form of phosphoric acid. Molybdic acid reagent is reduced by hydroquinone in the presence of phosphoric acid and the blue color developed is proportional to the amount of phosphorus present.

Technic.—

Serum or plasma must be used.

Place in a test tube 2 c.c. serum or plasma.

Add 4 c.c. distilled water (this is double volume).

Add 4 c.c. 20% trichloroacetic acid (double the volume of blood).

Shake vigorously and allow to stand ten minutes.

Filter through dry, ashless filter paper into a test tube.

In a sugar tube, marked "U" for unknown, place

3 c.c. filtrate (containing 0.6 c.c. serum).

Add 5 c.c. distilled water.

In a sugar tube, marked "S" for standard, place

3 c.c. standard phosphate solution (containing 0.025 mg. P).

Add 5 c.c. distilled water.

Add to both tubes

1 c.c. Benedict's hydroquinone-bisulphite reagent
and 1 c.c. Benedict's molybdic acid reagent.

Place both tubes, loosely stoppered, in a beaker of boiling water for ten minutes. Cool, and read in the colorimeter.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.025 \times \frac{100}{0.6} = \text{mg. of phosphorus in 100 c.c. serum or plasma.}$$

Example.—

Reading of standard is 15; reading of unknown is 15; 0.6 c.c. blood used.

$$\frac{15}{15} \times 0.025 \times \frac{100}{0.6} = 4.1 \text{ mg. phosphorus per 100 c.c. blood serum or plasma.}$$

Normal phosphorus is 3.7 mg. for adults; slightly above 5 mg. for children and infants.

Method of Fiske and SubbaRow**Reagents.—****10 N Sulphuric Acid.—**

To 1,300 c.c. distilled water add

450 c.c. concentrated sulphuric acid, c. p., slowly and with constant cooling of the flask.

Molybdic Acid Solution.—

Dissolve 25 gm. of ammonium molybdate, c. p.
in 200 c.c. distilled water.

Rinse into a liter volumetric flask containing 300 c.c. of 10 N sulphuric acid solution.

Dilute to 1000 c.c. with distilled water, and mix.

Trichloroacetic Acid, 10 Per Cent Solution.—

Dissolve 10 gm. trichloroacetic acid in 50 c.c. distilled water in a 100 c.c. volumetric flask and dilute to 100 c.c. with distilled water.

Stock Phosphate Solution.—

Dissolve 0.3509 gm. of pure monopotassium phosphate (KH_2PO_4) in distilled water in a liter volumetric flask and dilute to 1000 c.c. with distilled water.

Add 10 c.c. chloroform as a preservative and keep in the refrigerator.

Standard Phosphate Solution.—

Transfer 10 c.c. of the stock phosphate solution to a 100 c.c. volumetric flask.

Add 80 c.c. of 10% trichloroacetic acid.

Dilute to 100 c.c. with distilled water.

5 c.c. equals 0.04 mg. of phosphorus.

Amino-Naphthol-Sulphonic Acid Reagent.—

Dissolve 30 gm. of sodium bisulphite (NaHSO_3)

1 gm. of crystalline sodium sulphite ($\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$)

(or 0.5 gm. of anhydrous sodium sulphite)

in 200 c.c. distilled water.

Add 0.5 gm. purified 1,2,4-amino-naphthol-sulphonic acid and stir thoroughly.

Preserve in a dark glass bottle. This reagent should be prepared fresh once a month. The sediment that forms will settle to the bottom and need not be filtered out if care is taken not to stir it up.

Technic.—

Either serum or plasma may be used in the test. There must be no hemolysis.

Place 8 c.c. of 10% trichloroacetic acid in a small Erlenmeyer flask.

While rotating the flask, add 2 c.c. of serum or plasma.

Stopper the flask and shake vigorously.

Filter through a Whatman No. 42 filter paper.

Into a test tube graduated at 10 c.c., labeled "U" for unknown, pipette 5 c.c. of the filtrate.

Place 5 c.c. of the standard phosphate solution (5 c.c. = 0.04 mg. phosphorus) into a similar tube labeled "S" for standard.

Add to both tubes 1 c.c. of molybdic acid solution.

Add to both tubes 0.4 c.c. of sulphonic acid reagent.

Dilute the mixtures in both tubes with distilled water to the 10 c.c. mark, mix thoroughly, and allow to stand for 10 minutes.

Compare in the colorimeter.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 4 = \text{Mg. of inorganic phosphorus in 100 c.c. blood.}$$

Normal phosphorus is 3.7 mg. for adults; slightly above 5 mg. for children and infants.

Serum Inorganic Phosphate, Bodansky²

This method for the determination of serum inorganic phosphate is a modification of the Kuttner-Cohen method,³ as modified by Kuttner and Lichtenstein⁴ and Raymond and Levene,⁵ with corrections of the errors caused by the presence of trichloroacetic acid, as in the determination of inorganic phosphate in serum, and by glycerophosphate, as in the phosphatase determination. With these errors corrected, the accuracy of the Kuttner-Lichtenstein method

²Bodansky, A.: J. Biol. Chem. 99: 197, 1932.

³Kuttner, T., and Cohen, H. R.: J. Biol. Chem. 75: 517, 1927.

⁴Kuttner, T., and Lichtenstein, L.: J. Biol. Chem. 86: 671, 1930.

⁵Raymond, A. L., and Levene, P. A.: J. Biol. Chem. 79: 628, 1928.

has been maintained by Bodansky. The proper correction has been made for the very considerable deviation from Beer's law.⁶ A table of corrected values corresponding to the colorimetric readings obtained under standardized conditions may be conveniently substituted for the formula suggested by Bodansky. Bodansky states that inorganic phosphorus values ranging, within one series, between 0.0120 and 0.0360 mg. may be determined with an accuracy of 1 to 2 per cent, with the employment of one standard, 0.02 mg. of P; the determination of values outside of this range involves an error of 3 to 5 per cent.

Reagents.—

Stock Solutions.—

10 N Sulphuric Acid. (See normal solutions, page 24.) Keep in the refrigerator.

Sodium Molybdate Solution, 7.5%.—

Molybdic acid (Eimer and Amend, T.P., Special, "ammonia- and phosphate free") ----- 90 gm.

5 N sodium hydroxide ----- 250 c.c.

Place in a 2 liter volumetric flask, and dilute to volume. Mix. The solution should be *faintly* alkaline to phenolphthalein. Let stand and decant for use. This solution is preferred to Kuttner and Lichtenstein's, which is prepared by dissolving sodium molybdate, because the latter has frequently given appreciably blue blanks.

Stannous Chloride Solution, 60%.—

Dissolve 15 gm. of stannous chloride (Eimer and Amend, T.P.) in concentrated hydrochloric acid in a 25 c.c. volumetric flask, and dilute to 25 c.c. with concentrated hydrochloric acid.

Let stand in the refrigerator. This solution must be made up monthly. Bodansky et al. found that more consistent results over a wider range of inorganic phosphate values could be obtained with this more concentrated reagent, in the use of which they followed Raymond and Levene.

Standard Phosphate Stock Solution.—

Dissolve 110 mg. of potassium acid phosphate (LaMotte buffer grade) in about 100 c.c. (or more) of distilled water in a 250 c.c. volumetric flask.

Add 1 c.c. concentrated sulphuric acid, c.p.

Dilute to 250 c.c. with distilled water.

10 c.c. contain 1 mg. of P.

Solutions for Use in Analysis.—

Molybdate Reagent.—

Cold 10 N sulphuric acid ----- 1 volume

7.5% molybdate, stock solution ----- 1 volume

Add quickly, while mixing.

Distilled water ----- 2 volumes

By omitting the water, a more concentrated reagent is obtained for use with 7 c.c. aliquots.

Make up daily. When the above precautions are not observed, the reagent may have a yellow tinge, resulting in an appreciably blue blank after the addition of stannous chloride.

Dilute Stannous Chloride Reagent.—

Place 150 c.c. distilled water in a 200 c.c. volumetric flask.

Add 1 c.c. of 60% stannous chloride solution.

Dilute to 200 c.c. with distilled water. Make up daily, and keep in a glass-stoppered bottle in the refrigerator between analyses.

⁶Bodansky, A., Hallman, L., and Bonoff, R.: Proc. Soc. Exper. Biol. & Med. 28: 762, 1931.

Standard Phosphate Solution.—

Place 10 c.c. stock solution in a 250 c.c. volumetric flask.

Dilute to 250 c.c. with distilled water.

Preserve with a drop of toluene.

5 c.c. contain 0.02 mg. of P.

Reagent Blanks.—

Trichloroacetic acid -----5 c.c.

Molybdate reagent -----4 c.c.

Dilute stannous chloride reagent -----1 c.c.

Mix. The resulting mixture is colorless, or at most faintly blue or green. The blank is not only a check upon the quality of the chemicals used but also upon the proper preparation of the day's molybdate reagent.

Technic.—**Preparation of the Filtrate.—**

Bodansky has used this method in the determination of "inorganic phosphate" of plasma or serum, and in the serum phosphatase determination. The filtrate for the determination of serum inorganic phosphate is prepared as follows:

Serum -----1 c.c.

5% trichloroacetic acid-----9 c.c.

Mix. Let stand a few minutes. Filter through No. 44 Whatman filter paper. A lower dilution may be used, if preferred, when inorganic P is expected to be less than 3 mg. per 100 c.c.

Analysis.—

A single series for a colorimetric comparison may conveniently contain twenty test tubes (18 or 20 mm. by 150 mm.), including two or three containing *one* standard (Bodansky prefers 0.02 mg. of P).

Make the standards and the unknown aliquots (containing between 0.012 and 0.036 mg. of P) up to 5 c.c., if necessary, with water.

Add 4 c.c. of the molybdate reagent to each tube. (When 7 c.c. aliquots are used, with 2 c.c. of the concentrated molybdate reagent, it is advisable to dilute the standard tubes to 7 c.c.)

Mix the contents of each tube by tapping.

Add 1 c.c. stannous chloride reagent.

Immediately mix by a single inversion.

Check two or three standards against each other, as a matter of routine, both at the beginning and the end of each series of comparisons. Record duplicate readings in the usual manner, setting the standard at 20 in the colorimeter.

Perform analyses in duplicate.

Calculations.—

The usual formulae for calculations do not include a correction for the deviation from Beer's law. This deviation, which amounts to 20 per cent of the difference between the P content of the unknown and of the standard, may be corrected for by the use of the formula

$$\frac{0.48}{\text{Reading of Unknown}} - 0.0040 = \text{mg. P in aliquot.}$$

For convenience, however, the values corresponding to any given reading have been calculated and tabulated (see Table 33). In serum analyses the aliquot value may be converted into mg. of inorganic P per 100 c.c. of serum by the use of the formula

$$T \times \frac{D}{V} \times 100 = \text{mg. P per 100 c.c.}$$

where T equals the Table 33 value of the aliquot, corresponding to the colorimeter reading, D equals the dilution of the serum in the filtrate (usually 10 in serum phosphate determinations and 20 in the phosphatase determinations), and V equals the volume of the aliquot, in c.c.

TABLE 33.—INORGANIC P IN ALIQUOT, AT STATED COLORIMETRIC READINGS, CORRECTED FOR DEVIATION FROM BEER'S LAW. 0.02 MG. STANDARD SET AT 20 MM.

MM.	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
mm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
8	0.0560	0.0552	0.0545	0.0538	0.0531	0.0525	0.0518	0.0512	0.0505	0.0499
9	493	487	482	476	471	465	460	455	450	445
10	440	435	431	426	422	417	413	408	404	400
11	396	392	389	385	381	377	373	370	367	363
12	360	357	353	350	347	344	341	338	335	332
13	329	326	324	321	318	316	313	310	308	305
14	303	301	298	296	293	291	289	287	284	282
15	280	278	276	274	272	270	268	266	264	262
16	260	258	256	254	253	251	249	248	246	244
17	242	241	239	237	236	234	233	231	230	228
18	227	225	224	222	221	220	218	217	215	214
19	213	211	210	209	207	206	205	204	203	201
20	200	199	198	197	196	194	193	192	191	190
21	189	188	186	185	184	183	182	181	180	179
22	178	177	176	175	174	173	172	171	170	170
23	169	168	167	166	165	164	163	163	162	161
24	160	159	158	158	157	156	155	154	153	152
25	152	151	151	150	149	148	147	147	146	145
26	145	144	143	143	142	141	140	140	139	138
27	138	137	137	136	135	135	134	134	133	132
28	132	131	130	130	129	128	128	127	127	126
29	126	125	124	124	123	123	122	122	121	121
30	120	119	119	118	118	117	117	116	116	115
31	115	114	114	113	113	112	112	111	111	110
32	110	110	109	109	108	108	107	107	106	106
33	105	105	105	104	104	103	103	102	102	102
34	101	101	100	100	100	099	099	098	098	098
35	097	097	096	096	096	095	095	095	094	094
36	093	093	093	092	092	092	091	091	090	090

Table 34 shows the application of these corrections in a series of analyses over the range recommended. Near the limits of the range indicated in Table 33 greater errors may be expected (3 to 5 per cent).

TABLE 34.—CORRECTIONS FOR DEVIATION FROM BEER'S LAW

KNOWN P	UNCORRECTED (C)*		CORRECTED (T)*	
	PHOSPHORUS	ERRORS	PHOSPHORUS	ERRORS
mg.	mg.	per cent	mg.	per cent
0.0330	0.0305	-8.1	0.0326	-1.3
275	263	-4.5	276	0.4
220†	220	0	224	1.8
206†	206	0	207	0.5
185†	186	0.5	183	-1.1
165	172	4.0	166	0.6
144	152	5.3	142	-1.4
124	137	9.4	124	0

*Solutions of potassium acid phosphate of known concentration were used. C, calculated from the readings of the unknown without corrections for deviation from Beer's law; T, Table 33 values, including corrections for the deviation from Beer's law. The errors were calculated from the ratio of known P to value found.

†If many similar series are analyzed the corrected values even within this range (90 to 110 per cent of standard) would show, in most cases, a smaller error than the uncorrected values, although the deviation within this range is small and in any one series the relation may be reversed, as above, by the error in colorimetric comparison.

Trichloroacetic Acid and Glycerophosphate Corrections.—When trichloroacetic acid is present alone or with glycerophosphate, as in the determinations of serum inorganic phosphate and serum phosphatase, respectively, further corrections are necessary. The concentration of these substances has been so adjusted in these analyses as to permit the use of a convenient correction factor. This factor is +1 per cent per c.c. of serum P filtrate used in the analyses, and +2 per cent per c.c. of phosphatase filtrate (maximum correction 10 per cent). (For greater precision, the following corrections may be used for trichloroacetic

acid **plus** glycerophosphate: for 1 c.c. of phosphatase filtrate or less, 3 per cent per c.c. for 2 c.c. of filtrate, a 5 per cent correction; for 3 and 4 c.c. of filtrate, 6 and 8 per cent respectively; for 5, 6, and 7 c.c. of filtrate, 10 per cent.*)

TABLE 35A.—CORRECTIONS FOR TRICHLOROACETIC ACID AND GLYCEROPHOSPHATE

KNOWN P	VOL- UME	TRICHLOROACETIC ACID ONLY				TRICHLOROACETIC ACID AND GLYCEROPHOSPHATE			
		UNCORRECTED		CORRECTED		UNCORRECTED		CORRECTED	
		VALUE T	ERROR	INORGANIC P	ERROR	VALUE T	ERROR	INORGANIC P	ERROR
mg.	c.c.	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
0.0330	6					0.0298	-11	0.0328	0.6
247		0.0234	-6	0.0248	0.4	226	- 9	249	0.8
206		192	-7	204	1.0	187	-10	206	0
165		152	-9	161	2.4	148	-12	163	1.2
124		114	-9	121	2.5	110	-13	121	2.5
0.0330	4	312	-6	324	1.9	300	-10	324	1.9
247		237	-4	246	0.4	229	- 8	247	0
220		212	-4	220	0	204	- 8	220	0
165		163	-1	170	3.0	151	- 9	163	1.2
137		134	-2	139	1.5	127	- 8	137	0
0.0247	2	240	-3	245	0.8	235	- 5	247	0
206		201	-2	205	0.5	194	- 6	204	1.0
165		161	-2	164	0.6	154	- 7	162	1.9
124		119	-4	121	2.5	115	- 8	121	2.5

TABLE 35B.—CORRECTIONS FOR TRICHLOROACETIC ACID AND GLYCEROPHOSPHATE IN
SERUM ANALYSES

FIL- TRATE	KNOWN INORGANIC P				CALCULATED INORGANIC P			
	SERUM FILTRATE		ADDED P	TOTAL P	UNCORRECTED		CORRECTED	
	VOL- UME	PHOS- PHORUS			VALUE T	ERROR	INORGANIC P	ERROR
	c.c.	mg.	mg.	mg.	mg.	per cent	mg.	per cent
A	5						0.0210	
	4						168	
	4	0.0168	0.0063	0.0231	0.0224	- 4.2	233	0.8
B	3	126	095	221	217	- 2.0	224	1.2
	7						229	
	6						199	
	6	197	032	229	206	-11.2	227	-0.8
	5	165	063	228	208	- 9.6	229	0.4
	3	099	126	225	212	- 6.1	225	0
C	2	066	158	224	214	- 4.7	223	-0.5
	5						235	
	5						238	
	5	237	066	303	273	-11.0	300	-1.0
	4	189	099	288	263	- 9.5	284	-1.4
	3	142	132	274	253	- 8.2	268	-2.3
	2	094	165	259	245	- 5.7	257	-0.8
	1	047	196	245	237	- 3.4	244	-0.4

Solutions of potassium acid phosphate of known concentration were used, containing in addition trichloroacetic acid, and trichloroacetic acid **plus** glycerophosphate, in concentrations equal to those used in serum inorganic phosphate and serum phosphatase determinations, respectively. Aliquots of 7, 5, 3, and 1 c.c. yielded similar results, which are omitted here to save space.

*Greater relative effects (and, of course, smaller absolute effects) are found when the smaller aliquots of glycerophosphate solution are used. All factors were determined in a large number of determinations for each concentration of phosphate and for each aliquot volume (and, therefore, for a varying quantity of trichloroacetic acid or trichloroacetic acid plus glycerophosphate). The errors were calculated from the ratio of known contents to value found (yielding the percentage correction to be applied to the latter). The data serving as the basis for the corrections are too numerous to be reported. Typical series of analyses are shown in Tables 35 and 36. Duplicate determinations would yield average values for each sample, generally with smaller errors than shown in the tables.

The correction for the deviation from Beer's law is included in the "uncorrected" values **T** (taken from Table 33). Filtrate A, prepared for a serum phosphate determination, contained trichloroacetic acid; Filtrates B and C, prepared for a phosphatase determination, contained glycerophosphate as well.

These corrections may be applied to the final value (inorganic P per 100 c.c.) or, as Bodansky did, to the value **T**. Some values, before and after correction, are given in Tables 35*A* and *B*.

TEST FOR MAGNESIUM IN SERUM

Modified Method of Denis*

Principle.—

The calcium is removed as calcium oxalate and the magnesium is precipitated as magnesium ammonium phosphate, which is then determined colorimetrically as a phosphate.

Reagents.—

Ammonium Phosphate-Ammonium Hydroxide Reagent.—

Dissolve 50 gm. of ammonium phosphate in distilled water in a liter volumetric flask. Before diluting to 1 liter, add 5 c.c. of concentrated ammonium hydroxide. Mix.

Concentrated Ammonium Hydroxide.—

Ammonia-Alcohol.—

750 c.c. of 95% alcohol.

150 c.c. distilled water.

10 c.c. concentrated ammonium hydroxide.

Dilute to 1,000 c.c. with distilled water. This is 75% alcohol containing ammonium hydroxide.

Molybdate I Reagent, Fiske-SubbaRow

Dissolve 25 gm. of reagent grade ammonium molybdate in 200 c.c. distilled water.

Place in a liter volumetric flask 500 c.c. of 10 N sulphuric acid.

Add the molybdate solution, and dilute with washings to 1 liter with distilled water. Mix. This reagent is stable indefinitely.

Standard Phosphate Solution.—

1 c.c. is equivalent to 0.01 mg. of magnesium.

Dissolve 0.560 gm. of pure, dry monopotassium phosphate in distilled water in a liter volumetric flask, and dilute to 1,000 c.c. with distilled water.

Add a few drops of chloroform to prevent growth of molds.

Dilute 10 c.c. of this stock solution to 100 c.c. in a liter volumetric flask.

Aminonaphtholsulphonic Acid Reagent.—

Place 195 c.c. of 15% sodium bisulphite solution in a glass-stoppered cylinder.

Add 0.5 gm. of 1,2,4-aminonaphtholsulphonic acid (Eastman Kodak Co.).

Add 5 c.c. of 20% sodium bisulphite.

Stopper and shake until the powder is dissolved.

If solution is not complete, add more sodium sulphite, 1 c.c. at a time, with shaking.

Avoid an excess.

Transfer the solution to a brown glass bottle and store in the cold. The solution is usable for about 4 weeks if kept cold.

15% Sodium Bisulphite.—

Place 30 gm. reagent grade sodium bisulphite in a beaker.

Add 200 c.c. distilled water. Stir to dissolve.

If turbid, allow to stand in a well-stoppered bottle for several days and filter. Keep well stoppered after filtration.

20% Sodium Bisulphite.—

Dissolve 20 gm. of reagent grade anhydrous sodium bisulphite in distilled water and dilute to 100 c.c. with distilled water. Filter if necessary. Keep well stoppered.

*J. Biol. Chem. 52: 411, 1922.

Technic.—

Precipitate the calcium from 2 c.c. of serum as in the procedure for calcium in serum.

After centrifuging, pipette 3 c.c. of the supernatant fluid into a 15 c.c. graduated centrifuge tube.

Add, with stirring, 0.5 c.c. of 5% ammonium phosphate-ammonium hydroxide solution.

Add 2 drops of concentrated ammonium hydroxide.

Let stand overnight.

Centrifuge.

Siphon off the supernatant fluid and wash the tube with 5 c.c. of a mixture of 1 part of concentrated ammonium hydroxide, sp. gr. 0.9, and 2 parts of distilled water.

Centrifuge and siphon off wash liquid.

Repeat the washing a second and third time.

Wash finally with 5 c.c. of the alcohol-ammonium mixture.

Again siphon and let stand in a warm place until the ammonia has volatilized off.

Add to the residue in the centrifuge tube 1 c.c. of molybdate I reagent.

Tap to dissolve.

Add 5 c.c. of distilled water and set aside.

Prepare a standard:

Place 1 c.c. of molybdate I reagent in a graduated tube.

Add 3 c.c. of standard phosphate solution.

Add 2 c.c. of distilled water.

For photometric measurement, prepare a blank by placing 1 c.c. of molybdate I reagent in a graduated tube and adding 5 c.c. distilled water.

Add to each graduated tube 0.4 c.c. of aminonaphtholsulphonic acid reagent.

Immediately add distilled water to the 10 c.c. mark.

Mix and allow to stand 5 minutes before reading in the colorimeter or photometer.

For photometric measurement, set the photometer to "O" density at 660 mμ with the blank.

Calculations.—

(1) For colorimetric measurement.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.03 \times \frac{100}{1.2} = \text{mg. magnesium per 100 c.c. serum.}$$

(2) For photometric measurement.—

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.03 \times \frac{100}{1.2} = \text{mg. magnesium per 100 c.c. serum.}$$

The value 1.2 represents the fact that 3 c.c. of supernatant fluid obtained from 2 c.c. of serum, after precipitating the Ca as oxalate, corresponds to 1.2 c.c. of the original serum.

Normally, about 1 to 3 mg. of Mg are present in 100 c.c. of serum, and about 1.6 mg. per 100 c.c. of blood.

CARBON DIOXIDE COMBINING POWER OF BLOOD PLASMA**Van Slyke Method****Reagents.—**

1% Alcoholic Phenolphthalein.—Page 1199.

N1/ Ammonium Hydroxide.—

N/1 Sulphuric Acid.—

These two normal solutions are made according to the method outlined on pages 23 and 24.

Caprylic Alcohol or Phenyl Ether.—

Five Pounds of Metallic Mercury, Redistilled.—

Keep the reagents in drop bottles. The majority of the mercury is in the apparatus, but a small amount is kept in a drop bottle for use in sealing the apparatus in one step in the test.

Technic.—

Instruct the patient to avoid muscular exercise for an hour or two before the blood is taken. About 6 to 8 c.c. blood are required for the test.

Withdraw from a vein with no more constriction than is necessary, and with the least possible exposure to air, 6 to 10 c.c. blood, placing it immediately in a bottle with enough dry potassium oxalate to make about 0.5%. Mix thoroughly until the blood is completely oxalated.

Within one-half hour after the time of the withdrawal of the blood, separate the plasma from the cells by centrifuging the blood about fifteen minutes at 1,500 revolutions per minute. Slight hemolysis does not affect results appreciably, but hemolysis should be avoided as much as possible by immediate centrifugalization. Decant the supernatant plasma into a clean dry test tube, and cork until it is ready for use.

Prepare the Van Slyke apparatus as follows.* In order to make the apparatus airtight, first grease the two stopcocks with petroleum jelly, and hold in place with rubber bands.

The carbon dioxide apparatus is held in a strong clamp, which is lined with rubber, and the lower stopcock is supported by an iron rod, which is also covered with soft rubber tubing.



Fig. 123.—CO₂ apparatus with mercury seal at the upper stopcock.

Keeping both stopcocks open, pour five pounds of metallic mercury into the mercury leveling bulb, allowing it to run into the apparatus until it is entirely filled with mercury. Take care that capillaries above the upper stopcocks are also filled with mercury. Then close the two stopcocks. There should be no air bubbles within the apparatus. Any air which collects can be driven out by raising the mercury leveling bulb and forcing the air out through the upper arm. The apparatus can be tested for air by allowing the mercury to run down and then forcing it up by raising and lowering the leveling bulb rapidly. If all air has been removed, a metallic click is heard.

When the air has been forced out, the leveling bulb is hung on an extension on a level with the lower stopcock. If the lower stopcock is open to the right, the fluid in the upper cup should drop into the apparatus. If the bulb is hung above the apparatus, the mercury should rise when the upper stopcock and the lower stopcock are opened. The position of the bulb above the apparatus is called **position 1**; on a level with the lower stopcock, **position 2**. If the bulb is hung on a hook below the apparatus, and the lower stopcock is left closed, the mercury should drop at equal levels in the two arms below the

*The apparatus used for this test is a rather complicated glass equipment and must be carefully handled to prevent breakage and to prevent freezing of the stopcocks. It should be carefully checked for leaks. When the test is concluded, the stopcocks should be removed from their normal positions and tied to the instrument with pieces of string. These stopcocks should be well greased before insertion. The entire apparatus, when not in use, should be covered, preferably with a cellophane cover.

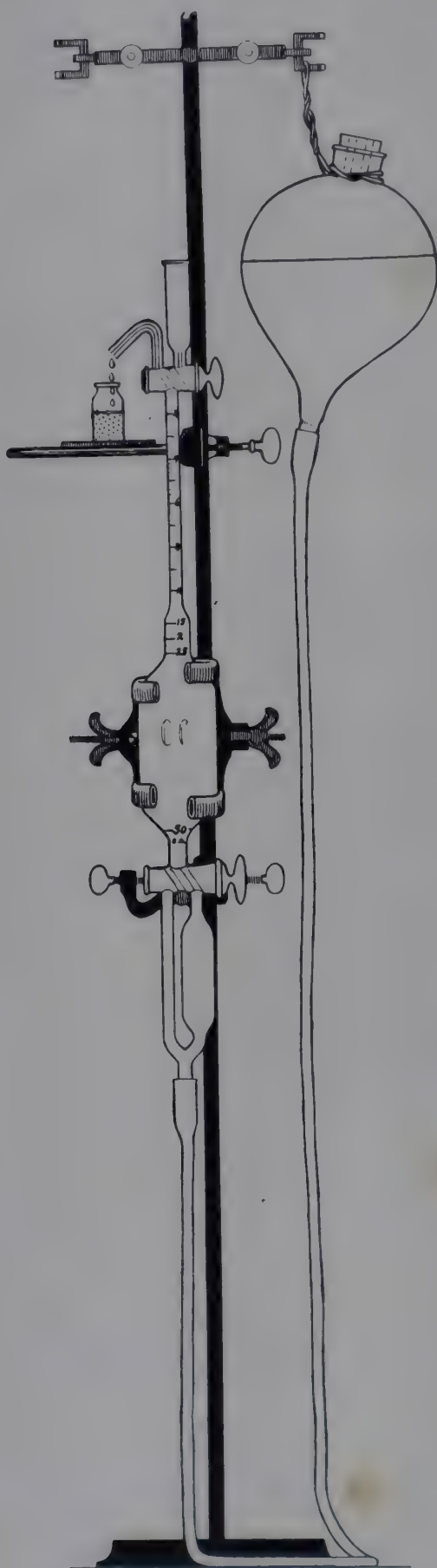


Fig. 124.

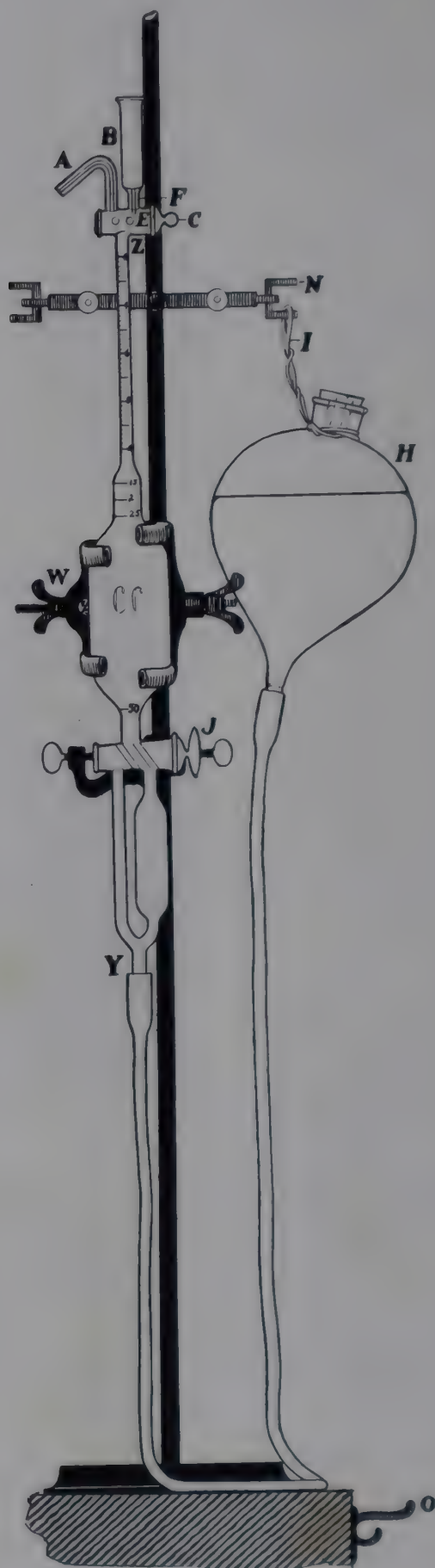


Fig. 125.

Fig. 124.—CO₂ apparatus with leveling bulb in position 1, forcing out the air.
 Fig. 125.—CO₂ apparatus with leveling bulb in position 2.

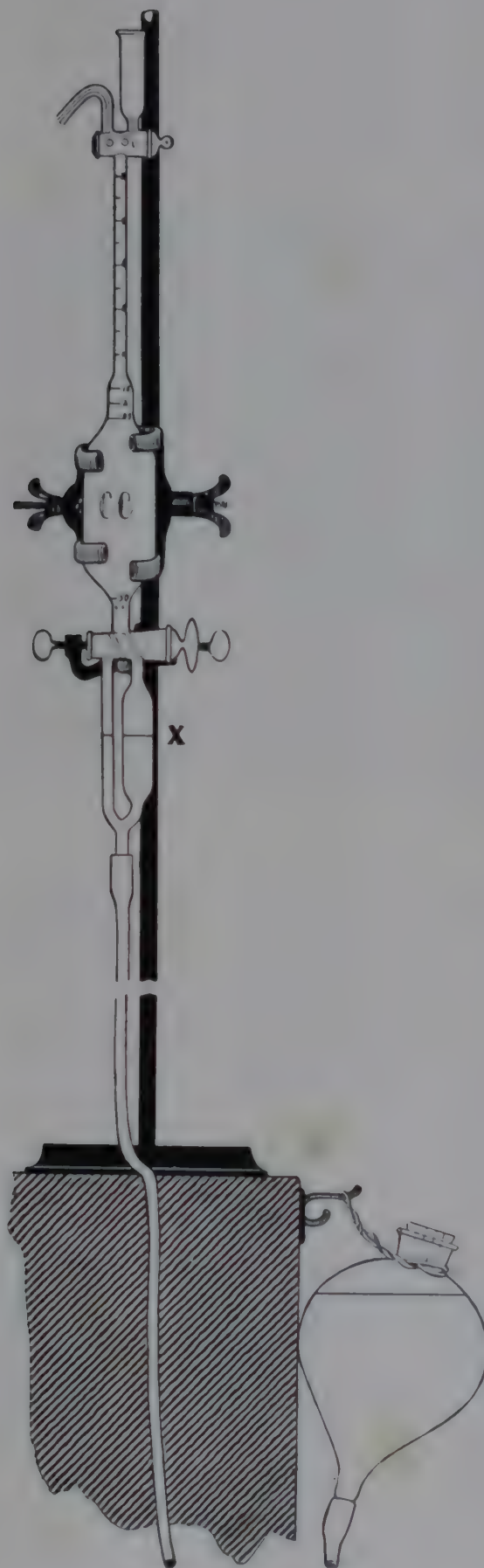


Fig 126.—CO₂ apparatus with leveling bulb in position 3. Mercury should not go below the mark X.

stopcock until it rests halfway down the arm. This is called **position 3**. If the bulb is hung in position 3, fluids may be admitted from the right arm of the instrument without entering the left side. The apparatus should always be tested before using, because the best results are obtained only if the positions of the bulb are correct.

After setting up the apparatus, and taking care to note that the upper stopcock is closed, hang the mercury bulb in position 2.

In order to determine the alkali reserve of the blood plasma, the plasma must be saturated with carbon dioxide at alveolar tension.

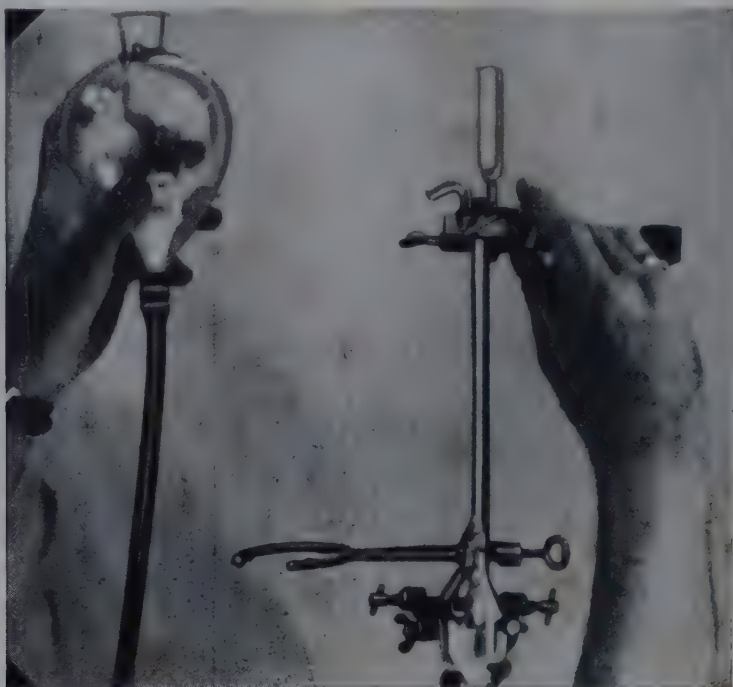


Fig. 127.—CO₂ apparatus showing operator forcing air out through the upper stopcock.

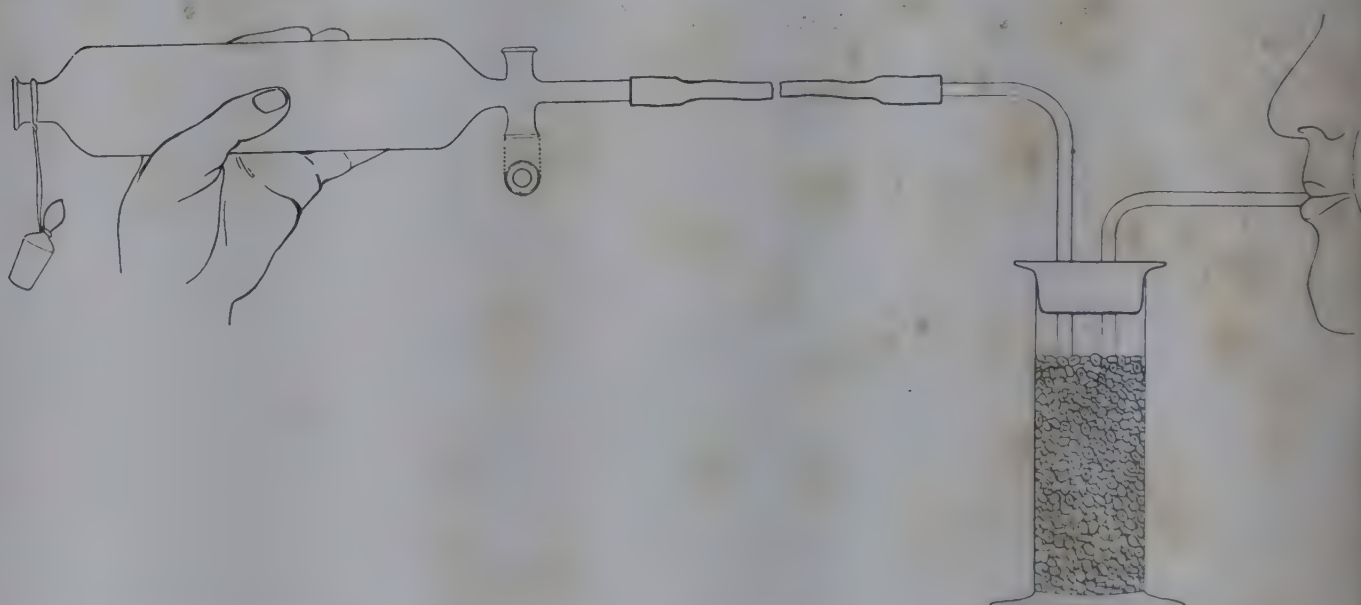


Fig. 128.—Saturating blood plasma with carbon dioxide. (Gradwohl and Blaivas.)

Method of saturating plasma with carbon-dioxide at alveolar tension:

Place 1.5 to 3 c.c. blood plasma in a 350 c.c. separatory funnel connected with a wash bottle filled with glass beads. Blow vigorously through the bottle of glass beads into the separatory funnel. If one blows directly into the separatory funnel, enough moisture collects on the walls of the funnel to appreciably dilute the plasma. By alternately opening and closing the stopcock of the separatory funnel, and shaking in between each successive blowing, the plasma can be saturated with carbon dioxide from the operator's breath. After blowing into the funnel through the bottle of beads, the stopcock and glass

stopper of the funnel are closed, and the plasma shaken for one minute. The saturating process should consume about three minutes. It is not possible to go past the saturation point.

Technic.—

Mercury bulb is in position 2, lower stopcock open to the right.

Place in the cup above the apparatus

1 drop of 1% alcoholic phenolphthalein.

Add 2 drops of N/1 ammonium hydroxide.



Fig. 129.—Adding reagents to the cup of the CO₂ apparatus.



Fig. 130.—CO₂ apparatus showing method of admitting fluid through the right arm of the lower stopcock.

If the solution does not turn deep pink, there is acid in the cup, and it must be cleaned before making the test. If the solution turns pink, proceed.

Add 0.5 c.c. distilled water, and mix thoroughly by drawing in and out of a dropper.

Withdraw all but two drops of the alkaline solution.

Place 1 c.c. of carbon-dioxide-saturated blood plasma under the alkali in the cup, taking care that none of the carbon dioxide escapes.

Let this mixture fall into the apparatus by slowly opening the upper stopcock to the right until only the top of the last drop is seen above the upper capillary of the cup; then close the upper stopcock.

Add 0.5 c.c. distilled water to the cup, and let this into the apparatus.

Add another 0.5 c.c. distilled water and let it into the apparatus.

Add 2 drops of caprylic alcohol

and 1.5 c.c. N/1 sulphuric acid to the cup, and mix thoroughly by drawing in and out of a dropper.

Let this fall into the apparatus until the meniscus of the mercury below the blood specimen stands at the mark 2.5 c.c. on the apparatus; then close the stopcock. Close the lower stopcock, also.

Draw off the excess sulphuric acid and caprylic alcohol mixture, replacing it with a few drops of mercury. Let the mercury into the apparatus until the capillary is filled. This step is to insure a seal of the apparatus against leakage of air into it.

Hang the mercury bulb in position 3.

Open the lower stopcock to the right and allow the mercury to fall in the apparatus until the top of the meniscus stands at the mark 50 c.c. Then quickly close the stopcock.

Hang the bulb in position 2 before proceeding with the next step.

Remove the apparatus from the stand, hold the finger over the cup, and hold the stopcocks in place, then mix by inverting 12 times. This liberates the carbon dioxide gas. During the process of lowering the mercury in the apparatus, bubbles of carbon dioxide can be seen rising. Replace the apparatus on the stand.

Again hang the mercury bulb in position 3.

Open the lower stopcock to the right, admitting all the fluid but none of the carbon dioxide. The time to close the stopcock is when the top of the fluid is halfway down the capillary opening of the stopcock.

Quickly close the stopcock.

Hang the mercury bulb in position 1.

Open the lower stopcock to the left, and allow the mercury to rise in the apparatus.

Remove the bulb from the hook, and move it up and down until the mercury in the bulb is at the same level as the mercury in the apparatus. At this point, the carbon dioxide in the apparatus is at atmospheric pressure.

Read from the graduations on the apparatus the height of the mercury.

To calculate the amount of carbon dioxide bound by the blood plasma, subtract 0.12 from the reading, and multiply the result by 100.

Example.—

Reading on the apparatus was 0.74.

$0.74 - 0.12$ equals 0.62 c.c. carbon dioxide bound by 1 c.c. blood plasma.

0.62×100 equals 62 c.c. carbon dioxide bound by 100 c.c. blood plasma or 62%.

Normal is 53% to 78% in an adult.

Figures lower than 50 per cent in adults indicate acidosis. The exact calculation of the result into terms of carbon dioxide bound as carbonate by the plasma is quite complicated, and consequently the worker is advised to subtract 0.12 from the reading on the apparatus. The result thus obtained gives approximately (within 2 to 3 per cent) the volume per cent of carbon dioxide bound by the plasma.

The **Van Slyke manometric blood gas apparatus** offers an improved method over that just described. This is the Fisher improved model of the Van Slyke blood gas apparatus. This apparatus is recommended for the rapid and accurate measurement of blood gases particularly, although it can be satisfactorily used in any determination in which the final product is a gas

suitable for measurement, or in which the final product will enter into a quantitative reaction producing a gas. The use of this apparatus greatly simplifies calculations, since the barometric pressure as a factor is eliminated.

The apparatus is constructed with the inlet capillary immediately below the stopcock, beveled at such an angle that the tendency of gas bubbles or droplets of mercury to cling to the sides is minimized. The accuracy of the 0.5 and 2 c.c. graduation marks is within the limits specified by Dr. Van Slyke. The rear half of the water jacket surrounding the extraction chamber is frosted and a lamp is located behind the jacket to produce an evenly

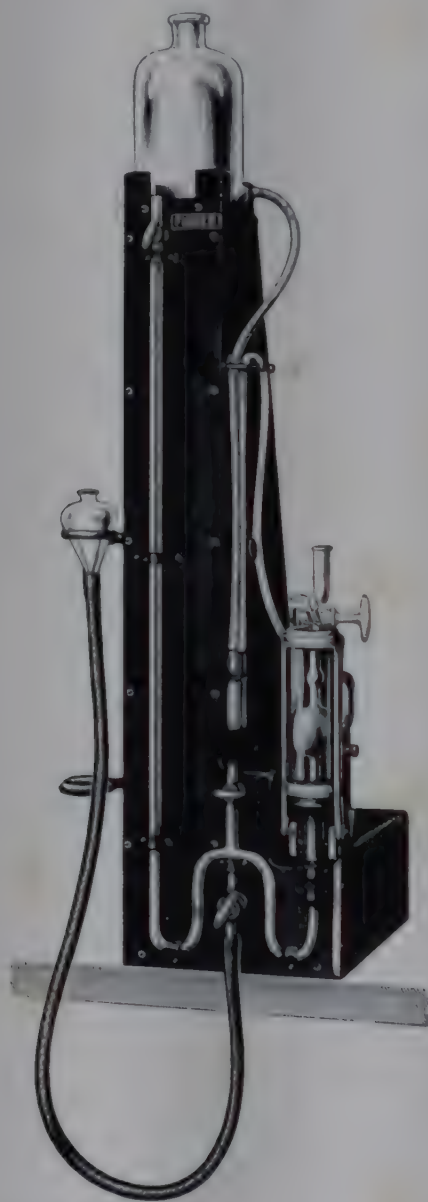


Fig. 131.—Van Slyke manometric blood gas apparatus. (Courtesy Fisher Scientific Co., Pittsburgh, Pa.)

diffused illumination. Volume readings within the reaction chamber are thus greatly facilitated. The manometer is graduated from 0 to 640 mm. and is mounted over a narrow slit on the front panel. The slit and manometer are illuminated from the rear by a 24-inch fluorescent lamp. The illumination thus provided is even throughout the entire length and is confined directly to the manometer.

All wooden parts are ebony finish and are built to withstand long, hard use. Suction and distilled water lines are equipped with pinchcocks and glass receptacles are provided on the apparatus to hold the glass delivery tubes when not in use. It is operated on 115 volt, 50 to 60 cycle AC.

The construction has been planned to place a minimum of strain on the glassware and prevent any tendency of the rubber connections to leak. The reaction pipette and its associated water jacket are firmly held within a cast aluminum bracket to which a

connecting rod is attached. The connecting rod is actuated by an excentric attached to a heavy base. A rheostat is mounted on the apparatus to control the speed of the motor, and consequently the shaking speed.

It is not necessary to dismantle the apparatus to remove the mercury or to clean it.

Carbon Dioxide Content of Serum, Plasma, or Whole Blood **Kopp-Natelson Microgasometer Method¹**

The Kopp-Natelson Microgasometer is a precision instrument used for measuring small gas volumes by the manometric method. Carbon dioxide, oxygen, carbon monoxide, nitrogen, etc., may be determined from volumes of blood as small as 0.03 c.c.

The Kopp-Natelson Microgasometer has several advantages over the hand-operated Van Slyke apparatus in the determination of carbon dioxide combining capacity or carbon dioxide content of serum: (1) only 0.03 c.c. is required for a valid determination, which eliminates QNS, especially from infants; (2) the determination is performed in a closed system, thus eliminating corrections for barometric pressure; (3) another advantage is the prevention of errors due to dissolved gases other than carbon dioxide, since this measurement is made by partial pressures. The rapidity with which the tests are performed, the mercury seals for prevention of air contamination, and the reproducibility of this method are further advantages of this apparatus.

Assemble the Microgasometer and fill (approximately 14 c.c. mercury) according to the directions which accompany the instrument.

Venous Blood.—

Draw blood under oil by syringe in the usual manner, allow to clot, loosen the clot, and centrifuge.

Transfer the serum to a small test tube containing 0.1 c.c. of *clean* mercury. The test tube is a 6 by 50 mm. culture tube without lip, scratched halfway down and broken to half its original size. It should be fire polished, cleaned with a detergent, rinsed, and dried before use. The capacity is about 0.5 c.c. Keep corked until ready for sampling.

If the serum and mercury are mixed, stable globules of mercury will appear. In such cases, centrifuge for a few minutes to break the emulsion.

Fingertip Blood.—

Fingertip blood contains the CO₂ content of arterial blood.

Puncture the skin, wipe off the first drop of blood, and allow the next drop to run into a narrow tube. This is ordinary Pyrex tubing of 1.5 mm. bore, which has been drawn out to a tip of approximately 0.8 to 1.0 mm. bore, and is approximately 10 cm. long. It should be lined with heparin, 3 mg. per c.c., by drawing up and expelling the heparin solution twice.

Hold the narrow tip horizontally to the blood and allow it to flow in by capillarity. Do not aspirate the blood into the tube.

When approximately 5 cm. of blood have entered the tube, seal the wide end with ordinary sealing wax or Dekhotinsky cement, using a flame. Press some of the wax into the tube to assure a seal.

Centrifuge, wax down, in an ordinary test tube.

Scratch above the cell level with a file and invert into a 0.5 c.c. test tube (described above); containing 0.1 c.c. mercury.

Break with a downward motion so that the plasma falls into the test tube and the cells remain in the original capillary. Shake or gently blow out any remaining plasma into the small test tube.

Keep the tube corked until ready for analysis.

¹Made by Kopp Scientific, Inc., 15 Park St., Springfield, Mass.

General Instructions.—

The sealing liquids, mercury, caprylic alcohol, mineral oil should be in containers similar to those of the reagents. (The 2 dram plastic screw-cap vials are supplied with the instrument.)

Open the reaction chamber stopcock. Advance the mercury slowly until a globule protrudes from the tip of the pipette. Immerse the pipette tip in the solution to be sampled.

Retreat with the aid of the vernier screw drive until the desired volume is reached.

Line up the apex of the mercury meniscus with the calibration mark on the pipette. If the sample to be measured is allowed to go beyond the mark, advance the vernier screw until it returns to the mark. An error may be introduced if the solution is allowed to go far beyond the mark. Although advancing the screw will bring the sample back to the mark, a small amount of liquid will adhere to the walls above the mark. This could become significant if the specimen is brought far beyond the mark. **CAUTION:** The vernier turns in a direction opposite the main dial.

The sample should never come in contact with air. Do not remove the pipette tip from the bottle containing the sample after the sample has been measured, but raise the bottle, or lower it, until the tip is in the sealing fluid. For example, for sampling water the tip is immersed in the mercury. Retreating with the screw will draw up mercury so that the water is trapped between the mercury at the tip and the mercury of the instrument. For sampling lactic acid, the tip is raised after sampling into the caprylic alcohol layer, and the caprylic alcohol is drawn into the instrument. In this way, the lactic acid is trapped between the caprylic alcohol layer and the mercury of the instrument.

If air is trapped in sampling, advance the screw to expel the air and repeat the procedure.

To sample several reagents in sequence, advance the screw until a small globule of the sealing liquid protrudes but does not fall off the tip of the pipette. Then immerse the pipette in the second reagent and draw the sample up, following by the sealing liquid, and proceeding as above. Repeat until all the reagents have been introduced into the pipette. Finally, draw mercury into the instrument beyond the reaction chamber stopcock, which is now closed, creating a mercury seal.

If a specimen is obtained under a sealing liquid which should not be introduced into the instrument in appreciable quantities because the gas liberated (CO_2) has some solubility in the oil, immerse the tip in the serum as before, with a mercury globule protruding. Advance the screw to allow several drops of mercury to fall off so as to be rid of oil adhering to the mercury, then measure the sample into the pipette by retreating with the screw.

Immerse the tip in the mercury at the bottom of the test tube and draw up a mercury seal. If the bottom of the tube contains cells or a clot, this procedure is not practical. In such cases, draw a small amount of mineral oil into the pipette, which serves as a seal. Wipe the pipette after removal of the tube, and then immerse in a pool of mercury. Advance the screw until all but approximately 1 mm. of oil remains at the tip. Draw the mercury up as the seal. The amount of mineral oil introduced in this way is less than 0.0003 c.c. and will not interfere with the determination.

Reagents.— **CO_2 Standard (50 Volumes per 100 c.c. [22.5 mM/L.]).—**

Dissolve 1.191 gm. sodium carbonate analytical reagent, anhydrous, dried at 100°C ., in distilled water, and dilute to 500 c.c.

Keep under mineral oil.

Transfer 2 c.c. to a small vial with plastic top.

Add 2 c.c. of clean, dry mercury, and cover with a layer of mineral oil for the working standard.

NOTE: CO_2 is given in vol. % or mM/L.

Lactic Acid, Approximately 1N.—

Dilute 90 c.c. of 85% lactic acid to 1 liter with distilled water.

Transfer 4 c.c. to a small vial and cover with 2 c.c. of caprylic alcohol,

Sodium Hydroxide, Approximately 1N.—

Dissolve 40 gm. of sodium hydroxide in distilled water and dilute to 1 liter.
Transfer 4 c.c. to a small vial and add 5 c.c. of *clean* mercury.

Water.—

Keep distilled water in a vial to which has been added 5 c.c. of *clean* mercury. Before using, boil and cool to eliminate any CO₂.

Waste Bottle.—

Keep a 50 c.c. Erlenmeyer flask handy for discharge of waste.

NOTE: Begin the determination with a globule of mercury suspended from the tip of the pipette. Always introduce the pipette into the sample or reagent vial with mercury globule suspended from the tip. This prevents air from entering the reaction chamber.

Principle.—

CO₂ is liberated from serum or plasma by the addition of lactic acid. The gas is shaken free under reduced pressure and the pressure (P₁) is measured at a constant volume. The CO₂ is reabsorbed in alkali and the pressure (P₂) of the residual gases is measured. (P₁ - P₂) in mm., multiplied by a factor (see Table 36), yields the CO₂ content of the plasma.

TABLE 36.—FACTORS FOR ESTIMATION OF CO₂ CONTENT

TEMPERATURE (° C.)	FACTOR (VOL. %)	FACTOR (mM/L.)
17	0.536	0.242
18	0.533	0.240
19	0.529	0.238
20	0.526	0.237
21	0.524	0.236
22	0.522	0.235
23	0.518	0.234
24	0.516	0.233
25	0.513	0.232
26	0.510	0.231
27	0.508	0.230
28	0.506	0.229
29	0.504	0.228
30	0.502	0.227
31	0.500	0.225
32	0.497	0.224

$$(P_1 - P_2) \times \text{Factor} = \text{CO}_2 \text{ content}$$

Example:

P₁ - 163 mm.

P₂ - 80 mm.

T - 28° C.

$$(163 - 80) \times 0.506 = 41.9 \text{ vol. \% of CO}_2$$

Technic.—

Introduce 0.03 c.c. of the sample according to the general rules, and follow with approximately 0.01 c.c. mercury seal from the same vial. The vial containing the sample also contains a layer of mercury on the bottom.

Introduce 0.03 c.c. of lactic acid, followed by 0.01 c.c. of caprylic alcohol seal, and 0.01 c.c. of mercury.

Introduce 0.1 c.c. of water followed by sufficient mercury to bring the mercury to the 0.12 c.c. mark beyond the stopcock, and close the stopcock in the reaction chamber.

Retreat the plunger until the mercury level is at the 3 c.c. mark in the reaction chamber. Shake with the shaking knob vigorously until all the mercury drops down into the 3 c.c. bulb, and the reagents are well mixed. This requires 10 seconds.

Allow to stand for 2 minutes with occasional shaking.

Bring the aqueous meniscus to the 0.12 c.c. mark with the plunger, and read pressure (P₁) in mm.

Release pressure by advancing plunger until the mercury in the manometer is at the manometer stopcock.

While holding the sodium hydroxide vial under the pipette, slowly open the reaction chamber stopcock. The mercury will move forward or back in the pipette slightly, depending upon the pressure. Advance the mercury so that a globule of mercury protrudes and introduce 0.1 c.c. of sodium hydroxide solution, followed by mercury to the 0.12 c.c. mark in the reaction chamber as previously described for water. Close the stopcock.

Bring the mercury down to the 3 c.c. mark and the aqueous meniscus promptly back to the 0.12 mark.

Read the pressure (P_2) in mm.

CAUTION: Never open the reaction chamber stopcock under vacuum or following a chemical reaction, without making sure that the aqueous column and the manometer mercury column are also up to the stopcock.

Calculation.—

$$(P_1 - P_2) \times \text{Factor} = \text{CO}_2 \text{ content.}$$

If it is customary to run a water blank, add all the reagents for estimating CO_2 , but substitute water for the unknown factor and take a reading. Add 0.1 c.c. of sodium hydroxide solution and take a second reading. The difference is the correction, C.

$$(P_1 - P_2 - C) \times \text{Factor} = \text{CO}_2 \text{ content.}$$

Cleaning.—

Release the pressure by advancing the plunger to bring the mercury to the *top of the manometer*. Open the reaction chamber stopcock, eject the aqueous contents and a little mercury into the waste bottle. Draw up 0.1 c.c. of water and retreat until the water is at the 3 c.c. mark in the reaction chamber but do not shake. Eject the water into the waste bottle. Draw up 0.1 c.c. of lactic acid and bring to the 3 c.c. mark. Shake for a few seconds and eject the lactic acid. Draw up 0.1 c.c. of water; bring to the 3 c.c. mark. Eject the water and the instrument is ready for the next determination. Now eject into the water or sodium hydroxide, whichever needs additional mercury, the excess mercury which has been introduced into the instrument.

When finished, the plunger should still have approximately 2 cm. for advancement. If the instrument is not to be used, withdraw the mercury from the tip of the pipette into the horizontal position (0.1 c.c. mark), leaving the reaction chamber stopcock open. This allows room for expansion in case of temperature changes.

Determination of Hydrogen Ion Concentration of Blood, Marriott, Levy, and Rowntree

Principle of the Method.—According to Marriott, Levy, and Rowntree¹ the indicator method has not proved of great value in the studies of hydrogen ion concentration of the blood, although the reaction of inorganic solutions may be determined accurately by this means.² Different indicators show their color changes at varying degrees of hydrogen ion concentration: for example, the color of methyl orange changes from pink to yellow as the pH of its solution changes from 3 to 5. At intermediate points, various colors may be obtained and a certain color indicates a definite pH. Similarly, phenolphthalein changes from colorless to pink between pH 8 and pH 10 and can be used for the measurement of H-ion concentrations between these two points. In carrying out the indicator method, it is necessary to have a series of standard solutions of known pH and an indicator exhibiting easily

¹Levy, Rowntree, and Marriott: Arch. Int. Med. 16: 389, 1915.

²Sörenson: Ergebn. d. Physiol. 12: 393, 1912. A full description of indicators as used for this purpose.

distinguishable color changes at hydrogen ion concentrations approximating that of the solution under consideration. It is then simply necessary to add equal amounts of indicator to the standard solutions and to the solution being tested and to determine which of the colors in the standard solutions most closely matches that of the unknown solution.

This method has been successfully used on the urine by Henderson and by Walpole. As proteins interfere with the colors of many indicators, and as both blood and serum possess color, it has been impossible to apply the method directly to the blood.

It seemed probable that the indicator method might be utilized for blood, provided coloring matters and proteins could be excluded by means of dialysis. If blood is dropped into collodion sacks and dialyzed for five minutes, the dialysate is free from proteins and coloring matter, but contains salts, and is well adapted to the use of indicators.

Since phenolsulphonephthalein exhibits definite variations in quality of color, with very minute differences in hydrogen ion concentration between pH 6.4 and 8.4, it was adopted as the indicator in this method.

Preparation of Standard Colors.—Standard phosphate mixtures are prepared according to Sörensen's directions as follows:

M/15 Acid or Primary Potassium Phosphate.

Dissolve 9.078 grams of the pure recrystallized salt (KH_2PO_4) in freshly distilled water and make up to one liter.

M/15 Alkaline or Secondary Sodium Phosphate.

Expose the pure recrystallized salt ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) to the air for from ten days to two weeks, protected from dust. Ten molecules of water of crystallization are given off and a salt of the formula $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ is obtained; dissolve 11.876 grams of this in freshly distilled water and make up to 1 liter. The solution should give a deep rose-red color with phenolphthalein. If only a faint pink color is obtained, the salt is not sufficiently pure.

Mix the solutions in the proportions indicated in Table 37 to obtain the desired pH:

TABLE 37

pH	6.4	6.6	6.8	7.0	7.1	7.2	7.3	7.4	7.5	7.6	7.7	7.8	8.0	8.2	8.4
Primary Potas. Phos. c.c.	73.0	63.0	51.0	37.0	32.0	27.0	23.0	19.0	15.8	13.2	11.0	8.8	5.6	3.2	2.0
Secondary Sodium Phos. c.c.	27.0	37.0	49.0	63.0	68.0	73.0	77.0	81.0	84.2	86.8	89.0	91.2	94.4	96.8	98.0

Place 3 c.c. of each of the solutions in suitable small test tubes (100 × 10 mm., inside measurement). Add five drops of an aqueous 0.01 per cent solution of phenolsulphonephthalein to each tube. Seal off the tops. The series of colors, representing different concentrations of hydrogen ions, constitutes the standards for comparison of color in carrying out the determination.

Preparation of Sacks.—Dissolve one ounce of collodion (Anthony's negative cotton) in 500 c.c. of a mixture of equal quantities of ether and ethyl alcohol. The solid swells up and dissolves with occasional gentle shakings in forty-eight hours. As a small amount of brown sediment separates out at first, the solution should stand for at least three or four days, after which the clear supernatant solution is ready for use. Fill a small test tube (120 by 9 mm., inside measurement) with this mixture, invert, and pour out half the

contents. The tube is then righted, and the collodion allowed to fill the lower half again. Invert a second time and rotate on its vertical axis, the collodion being drained off. Care must be taken to rotate the tube, in order to secure a uniform thickness throughout. Clamp the tube in the inverted position and allow to stand for ten minutes, until the odor of ether finally disappears. Fill it five or six times with cold water, or allow it to soak five minutes in cold water. Run a knife blade around the upper rim, so as to loosen the sack from the rim of the test tube, and run a few cubic centimeters of water down between the sack and the glass of the tube. Extract the tube by gentle pulling, after which preserve by complete immersion in water.

Salt Solution.—Dialyze the blood or serum against an 0.8% sodium chloride solution.

Before applying the test, it is necessary to ascertain that the solution is free from acids other than carbonic. To determine this, place a few cubic centimeters of the salt solution in a Pyrex test tube and add one or two drops of the indicator, whereupon a yellow color will appear. On boiling, carbon dioxide is expelled, and the solution loses its lemon color and takes on a slightly brownish tint. In the absence of this change, other acids are present, and the salt solution is therefore not suitable. If, on the other hand, on adding the indicator, pink at once appears, the solution is alkaline and hence cannot be used.

Technic.—

The technic can be carried out on serum, plasma, or whole or defibrinated blood. The work must be done in a room free from fumes of acids or ammonia.

Run 1 to 3 c.c. of clear serum or of blood, by means of a blunt pointed pipette, into a dialyzing sack which has been washed inside and outside with salt solution and which has been tested for leaks by filling with the salt solution. Lower the sack into a small test tube (100 by 100 mm., inside measurement) containing 3 c.c. of the salt solution, until the fluid on the outside of the sack is as high as on the inside. Allow from five to ten minutes for dialysis. Remove the collodion sack and add 5 drops of the indicator thoroughly mixed with the dialysate. Then compare the tube with the series of standards until the corresponding color is found, which indicates the hydrogen ion concentration present in the dialysate.

These tests have been carried out with 3 c.c. of blood or serum. The same results are obtained with 1 c.c. of blood or serum on the inside of the sack and with this amount it is immaterial whether there is 1 or 3 c.c. of salt solution on the outside.

Comparison of Tubes With Standards.—For this, a good light (natural or artificial) and a white background are requisites. Readings must be made immediately. The tube matching most closely is selected and also the tubes on either side of it. These are critically inspected against a white background. Changing the order of the tubes often makes differences more apparent.

Controls of the Method.—Repeated duplicate determinations on the same samples of blood and of serum convinced Marriott and his co-workers that the limits of error are very slight: for example, the serum from a case of mild acidosis (using quantities of serum varying from 1 to 3 c.c. and dialyzing for from five to fifteen minutes) gave the following series of readings: 7.55, 7.55, 7.55, 7.55, 7.6, 7.55, 7.55, 7.55, 7.55, 7.55. The oxalated whole blood from the same case gave the following readings under similar conditions: 7.25, 7.25, 7.25, 7.25, 7.2, 7.25, 7.25, 7.3, 7.25, 7.25, 7.25, 7.25, 7.25.

In order to test out the effect of the variations in the sacks used, a number of determinations were made on the same sample of serum with the following results: ordinary thin sack, 7.7; thick sack, 7.7; opaque, irregular sack, 7.7; ordinary thin sack, 7.65; a very thick sack, 7.7. A series of six normal sera was run through, 3 c.c. and 1 c.c. portions being used for dialysis. In every instance identical readings were obtained.

A brief word of explanation may be given for those unaccustomed to the physicochemical methods of expressing the reaction of a solution. A solution is acid when it contains an excess of hydrogen over hydroxyl ions, neutral when hydrogen and hydroxyl ions are in equal numbers, and alkaline when hydroxyl ions predominate. An acid of "normal" strength contains, in one

liter, a gram of hydrogen capable of forming hydrogen ions, and its strength may be expressed as 1 N. Diluting such a solution ten times results in 1/10 N or a solution containing 1/10 gram of actual or potential hydrogen ions to the liter. Continuing the process of dilution until 1/10,000,000 normal acid is obtained, in such a solution there would be 1/10,000,000 gram of hydrogen ions. Pure water, however, dissociates to form hydrogen and hydroxyl ions, and at 20° C. contains approximately 1/10,000,000 gram of hydrogen ions to the liter and an equivalent amount of hydroxyl ions. That is to say, pure water, our standard of neutrality, is 1/10,000,000 N acid and also 1/10,000,000 N alkali. To avoid writing large figures it is customary to use the logarithmic notation and to express 1/10,000,000 N as 10^{-7} N or more conveniently, as suggested by Sörensen, to drop the 10 and minus sign and say pH 7. If there is less than 1/10,000,000 gram of hydrogen ions in one liter, the solution is less acid than water, that is, it is alkaline—so, pH 8 means actually 1/1,000,000 N alkali. The higher the exponent, the more alkaline, or what is saying the same thing, the less acid the solution.

pH 1 = N/10 acid.

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pH 6 = N/1,000,000 acid.

pH 7 = NEUTRALITY.

pH 8 = N/1,000,000 alkali.

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pH 14 = N/10 alkali.

The reaction of the blood serum varies approximately between pH 7 and pH 8, the neutral point, pH 7, being reached only in severe uncompensated acidosis, and a reaction of pH 8 being attained perhaps only after administration of alkalies.¹

LaMotte Blood pH Outfit²

This is a method for the accurate and rapid estimation of the reaction of the blood in acidosis and alkalosis by the pH method. The test is made upon the blood plasma, wherein the CO₂ is retained by collecting and diluting the specimen under oil. Phenol red indicator is added to the plasma which has been diluted with physiologic salt solution, and the color that is exhibited by the indicator is then compared with the blood pH color standards. The pH color standards are prepared in intervals of 0.05 pH, and readings may be made with an accuracy of within 0.03 pH unit.

Determination of the Alkali Reserve of the Blood Plasma, Marriott

Marriott³ published a method which gives the hydrogen ion concentration of the dialysate of blood serum after removal of the carbon dioxide, that is in a measure a modification of the indicator analysis of the preceding test, but is more accurate and gives more information than that method. This method serves for the detection and accurate quantitative estimation of the degree of the acidosis.

¹The apparatus and reagents for making this test can be obtained in convenient form from Hynson, Westcott & Dunning, Baltimore, Md.

²Cullen: J. Biol. Chem. 52: 501, 1922.

³Marriott: Arch. Int. Med. 17: 840, 1916.

Apparatus Required.—Set of tubes containing standard phosphate mixtures; a solution of phenolsulphonephthalein in 0.8 per cent sodium chloride; collodion sacks; pipette to measure 0.5 c.c.; small test tubes for dialyzing and aerating; atomizer bulb; glass tube or pipette drawn out to a fine capillary point; color comparison box.

Preparation of Phosphate Mixtures.—

M/15 Acid Potassium Phosphate.—

Dissolve 9.078 gm. of the pure recrystallized salt (KH_2PO_4) in freshly distilled water. Add 200 c.c. of 0.01 per cent phenolsulphonephthalein and make up the whole to 1 liter with distilled water.

M/15 Alkaline Sodium Phosphate.—

Expose the pure, recrystallized salt ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) to the air for from ten days to two weeks, protected from dust. Ten molecules of water of crystallization are given off and a salt of the formula $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ is obtained. Dissolve 11.876 gm. of this salt in distilled water. Add 200 c.c. of 0.01 per cent phenolsulphonephthalein and make up the whole to one liter. The exact amount of indicator is immaterial, provided the same amount of indicator is added to each of the phosphate solutions, and a corresponding amount is added to the salt solution, to be subsequently described. Add a small crystal of thymol to each solution to prevent the growth of molds. The solutions should be preserved in Pyrex or Non-sol glass vessels. Mix the solutions in the proportions indicated below to obtain the desired pH.

pH	7.0	7.2	7.4	7.6	7.8	8.0	8.2	8.4	8.6
KH_2PO_4 , c.c.	37.0	27.0	19.0	13.2	8.8	5.6	3.2	2.0	1.0
Na_2HPO_4 , c.c.	63.0	73.0	81.0	86.8	91.2	94.4	96.8	98.0	99.0

Place these solutions in small test tubes, approximately 100 mm. long by 8 mm., internal diameter, of glass that does not readily give off alkali. The tubes are stoppered or sealed off. They should be kept in a dark place when not in use. Under these conditions, the solutions retain their colors for long periods of time.

Preparation of Salt Solution.—

Dissolve 8 gm. of chemically pure sodium chloride in distilled water. Add 220 c.c.¹ of 0.01 per cent phenolsulphonephthalein solution and make up the whole to one liter with distilled water. The solution should contain no free alkali and no acid other than carbonic. Test the solution by boiling a little of it for a minute or so in a Pyrex glass test tube, in order to expel carbonic acid.² Cool the solution quickly under the tap and compare with the phosphate standards. Its reaction should be 7.0. If the reaction differs from this, it may be corrected by the addition of a few drops of very dilute acid or alkali to the whole solution. The salt solution must be kept in a vessel of Pyrex or Non-sol glass, or in a vessel of ordinary glass that has been well paraffined on the inside.

Method of Determination.—The determination must be carried out in a room free from acid or ammonia fumes. Serum, oxalated plasma, or blood may be used. Serum is preferred, as the addition of oxalate, unless exactly neutral, introduces a source of error. The blood should be collected in a small tube and the serum separated as quickly as possible, preferably by centrifuging.³ Hemolysis must be avoided.

Pipette exactly 0.5 c.c. of serum into one of the small collodion sacks, which has previously been washed inside and out with the salt solution.⁴ Lower the sack into a

¹The concentration of indicator in the salt solution is purposely made 10% greater than in the phosphate mixtures, as during the dialysis a certain amount of indicator is lost by passing into the sack.

²If boiled in a soft glass tube, alkali is given off from the glass and the solution is colored pink. Instead of boiling to remove carbon dioxide, the solution may be aerated with a current of air that has been freed from carbon dioxide by passing through a strong solution of sodium hydroxide.

³If carbon dioxide escapes from the plasma as a result of shaking or allowing the blood to remain exposed to the air, a passage of alkali from the plasma into the cells occurs with a resultant slight diminution in the alkali reserve of the plasma. Once the plasma or serum is separated from the corpuscles, loss of carbon dioxide is without effect on the alkali reserve.

⁴In washing the sack, no part but the top edge should be touched with the fingers. The sack is emptied by tipping it with a clean glass rod or with a microscope slide. Sacks may be used more than once, provided they are thoroughly washed with salt solution after each test.

small test tube, approximately 8 mm. internal diameter and 50 mm. long, containing 2 c.c. of the indicator salt solution. The level of the fluid on the outside of the sack should be at least as high as that on the inside. At the end of seven minutes remove the sack and transfer the dialysate to a clean test tube 100 to 140 mm. long and having the same diameter as the tubes containing the phosphate standards. A rapid current of air is bubbled through the solution in order to remove carbon dioxide. This is accomplished by means of an atomizer bulb connected with a narrow glass tube drawn out to a capillary point. The air current should be as rapid as possible without blowing liquid out of the test tubes.¹ Continue blowing for three minutes and then compare the color in the tube with that in the standard phosphate tubes, interpolating when necessary. The reading is a measure of the reserve alkalinity. For convenience of expression this value is referred to as the "RpH" of the serum, to differentiate it from the "pH" as determined in the method previously described by Levy, Rowntree, and Marriott.

Results Obtained.—Normal Individuals. The sera of a large number of normal adults were examined by the method described. In every instance the RpH was found to be 8.5 ± 0.05 , provided the subjects examined were on a general mixed diet. A normal adult's serum drawn after a fast of sixteen hours gave a reading of 8.35. The sera of infants gave values slightly lower than those of adults. For normal infants under one year of age, a value of 8.3 for the RpH of the serum was not infrequently encountered. This may be due partly to the fact that infant's blood is often obtained by cupping; the lower value, however, is more likely an evidence of the tendency toward acidosis that is known to be present in infants.

This accords well with the observed fact that the carbon dioxide tension in the alveolar air of infants is lower than that of adults, and that the combined carbon dioxide of the plasma is less in infants and that the ammonia coefficient in the urine is often higher. This slight acidosis might well be the result of the more active metabolism of infants, leading to a proportionately greater production of acids.

LaMotte Alveolar CO₂ Tension Outfit²

This set is used for determining the carbon dioxide tension of alveolar air as an aid in the diagnosis of suspected and positive cases of acidosis. By rebreathing air under certain definite conditions a sample is obtained the carbon dioxide tension of which is virtually that of venous blood. The analysis of the sample of alveolar air depends upon the fact that if it is passed through a solution of sodium bicarbonate containing a suitable chemical indicator the final solution will contain sodium bicarbonate and dissolved carbon dioxide in definite ratio, depending upon the carbon dioxide present in the sample of alveolar air. The color of the indicator will change in accordance with the carbon dioxide tension. Comparison is made against permanent color standards.

Estimation of Alkali Reserve Using Small Amounts of Plasma Method of Lehmann³

This method is based on the methods of Wootton and King⁴ for the titration of alkali reserve for small amounts of plasma.

¹Foaming rarely occurs. It may be present as a result of allowing some serum to spill over the outside of the sack. In case foaming is great enough to be troublesome, it may be prevented by adding a drop of octyl alcohol or toluol.

²Marriott: J. A. M. A. 66: 1594, 1916.

³Lehmann, H.: Lancet, p. 718, March 31, 1951.

⁴Wootton, I. D. P., and King, E. J.: Biochem. J. 44: 12, 1949.

Apparatus.—

Dreyer's tubes carefully washed with distilled water and dried. The tubes should not be of the very small variety, and should have sizable rims.

Dreyer's tube rack with holes labeled 16, 18, 20, up to 36.

Screw-capped airtight ointment jars, 6 to 7 cm. in diameter, 6 to 7 cm. high, labeled 16, 18, 20, up to 36.

Graduated 1 c.c. pipettes.

Reagents.—

N/10 Sulphuric Acid.—See page 24.

Dilutions of Sulphuric Acid.—These dilutions are given in the third column of Table 38. Prepare the various dilutions and store in waxed or hard glass bottles. Label: 16, 18, 20, up to 36.

Methyl-Red Indicator, Certified, Water-Soluble.—

Sulphuric Acid-Methyl Red Mixture.—

Prepare the 11 dilutions of sulphuric acid as given in Table 38, as stated above. They produce a pH of 5.5 when mixed with equal amounts of plasma varying in alkali reserve from 16 to 36 meq./L. of bicarbonate.

Prepare 6 Dreyer tubes for each dilution of the acid and place them in the correspondingly labeled screw-capped ointment jars.

TABLE 38.—DILUTIONS OF SULPHURIC ACID

A pH of 5.5 is achieved by mixing equal quantities of sulphuric acid and plasma (saturated with alveolar carbon dioxide) under the following conditions:

PLASMA OR SERUM		STRENGTH OF SULPHURIC ACID REQUIRED (C.C. OF N/10 ACID PER 100 C.C.)
MEQ. OF BICARBONATE PER LITER	CO ₂ COMBINING POWER (VOL. %)	
16	35	30.3
18	40	34.0
20	44	37.9
22	48	41.7
24	53	45.4
26	57	49.0
28	62	52.9
30	66	56.7
32	70	60.6
34	75	64.2
36	79	68.0

To do this, fill each tube with 0.2 c.c. of methyl red indicator and 0.1 c.c. of the appropriate acid.

Mix by rolling the tubes between both hands.

Methyl red is chosen because it shows a distinct color change from red to orange-yellow when the acid is neutralized beyond pH 5.5; it also has a particularly low salt and protein error.

Store the tubes with the mixtures of acid and indicator in the jars until needed. They are stable for 1 or 2 weeks, if kept in a dark place. After about a week, some of the methyl red may become converted into a brownish precipitate. This does not seriously interfere with the color changes on neutralization; the yellow color of a solution with a pH more than 5.5 is less clear and slightly brown, but can still be well distinguished from red. If there is any doubt, the addition, from a capillary pipette, of a drop of water-soluble methyl red indicator will emphasize the red and yellow colors.

Technic.—

Place 1 Dreyer tube from each container in a rack with holes labeled 16 through 36.

Saturate plasma with alveolar air by breathing through it from a 1 c.c. graduated pipette. Use this pipette for adding 0.1 c.c. of plasma to each tube.

Mix by rolling the tubes between both hands.

After 20 seconds, note which mixtures appear clearly yellow or orange, and in which tube the fluid retains the last definite tinge of red. The number of this last tube corresponds to the alkali reserve of the plasma.

If the red is deep and the preceding tube is clearly yellow, the value can be interpolated; e.g., if tube 28 is deep red and tube 26 clearly yellow, the alkali reserve is 27 meq. of bicarbonate per liter. If tube 28 is red and 26 is orange-yellow, the alkali reserve is 28 meq./L.

To overcome difficulty in distinguishing between orange-yellow and red, add in two Dreyer's tubes 0.1 c.c. of indicator to 0.3 c.c. of certified potassium hydrogen phthalate buffer of pH 5.5 and pH 5.6 and compare the two resulting colors. Or, a certified methyl red capillator card with capillary pipettes might help to establish in which mixture the tinge corresponding to pH 5.5 is reached. Or, add from a capillary pipette a drop of dilute indigo-carmin solution which will help distinguish more clearly between a brownish color at pH 5.6 and a wine-red tinge at pH 5.5. If there is any doubt, it will be safe to interpolate between the definitely red and possibly red tubes. As the difference between two tubes corresponds to 4.4 volumes of CO_2 per 100 c.c., the result will differ from the correct reading at the most by a combining power of 2.2 volumes of CO_2 per 100 c.c.

In emergencies, or when only a little plasma is available, only three tubes need be tested, corresponding to 22, 26, and 30 meq. of plasma bicarbonate per liter or a CO_2 combining power of 48, 57, and 66 volumes per 100 c.c. The last value is kept relatively low for the detection of alkalosis in infants. The screw-capped containers in which these three different sets of tubes are kept can be labeled as follows:

- (1) 22 meq./L.: Add 0.1 c.c. of plasma.
If it turns yellow—no severe acidosis.
If it remains red—severe acidosis.
- (2) 26 meq./L.: Add 0.1 c.c. of plasma.
If it turns yellow—no acidosis.
If it remains red—acidosis.
- (3) 30 meq./L.: Add 0.1 c.c. of plasma.
If it turns yellow—alkalosis.
If it turns red—no alkalosis.

SERUM BICARBONATE, BEDSIDE DETERMINATION

Method of Scribner and Caillouette¹

Principle.—

This is an adaptation of the titrimetric procedure of Van Slyke and others. It is made by adding a measured excess of standard acid to a sample of plasma or serum. The acid reacts with the bicarbonate and converts it to carbon dioxide, which is allowed to escape. The specimen is then titrated with a standard alkali back to the original pH of 7.4; the effect of the serum buffers on the acid is thus cancelled out. The end point is determined by comparing the sample with a permanent color standard, an aqueous buffer at pH 7.4, which contains the same concentration of phenol red as the test.

Solutions are measured with tuberculin syringes, and reagent strengths are adjusted so that the result can be read directly from the scale of the syringe.

Reagents.—

Standard Carbonate Solution, 25 meq./L.—

Dissolve 1.32 gm. of dry, reagent grade, anhydrous sodium carbonate (Na_2CO_3) and 5.85 gm. of dry sodium chloride in distilled water, and dilute to 1 liter with distilled water.

The inclusion of sodium chloride makes this solution suitable for the standard in the chloride and total base procedures.

This reagent is used to standardize the bicarbonate procedure.

¹Scribner, B. H., and Caillouette, J. C.: J. A. M. A. 155: 644, 1954.

Standard N/10 Nitric Acid.—

Dilute 7 c.c. of concentrated nitric acid to 1 liter with distilled water.

Using a volumetric pipette, measure 4 c.c. of standard carbonate solution into the titrating test tube.

Add 1 drop of indicator (methyl orange) with an end point in a pH range of 4.0 to 5.0.

Titrate the carbonate sample with this roughly diluted nitric acid to the first definite color change toward amber red, using a tuberculin syringe or buret.

Dilute the nitric acid until 1 c.c. reacts exactly with 4 c.c. of the standard carbonate solution.

Standard N/10 Sodium Hydroxide.—

Prepare a saturated solution by dissolving 110 gm. of sodium hydroxide in 100 c.c. of distilled water.

Allow to stand 2 to 3 days until the carbonate precipitates. If time is limited, pellets from a freshly opened bottle of reagent grade sodium hydroxide may be used. Carbonate contamination will be greater with this method, however.

Dilute 7 to 8 c.c. of this strong base in about 1 liter of distilled water that has been boiled or aerated to remove excess carbon dioxide. If this reagent is used also for the total base determination, it must be made in 3.6% sodium nitrate.

Standardize against the N/10 nitric acid, using methyl orange indicator.

Store in a tightly capped bottle, preferably polyethylene, taking care to avoid exposure to carbon dioxide.

Discard the supply in the test set when the 2 oz. bottle becomes one-half to two-thirds empty. Then rinse the bottle twice with fresh base, and refill. This prevents the accumulation of carbonate precipitate.

Indicator.—

Dissolve 400 mg. of phenol red, water-soluble sodium salt, in 1 liter of distilled water. A 0.04% aqueous solution of phenol red or the water-soluble sodium salt may be purchased from laboratory supply houses.

Equipment.—**Sample Syringe.—**

A 1 c.c. tuberculin syringe may be fitted with a 1½ inch, 20 gauge needle in the usual manner. However, it is recommended that the shaft be cemented into the tip as follows:

Cut off the hub, and square the ends of the shaft; be sure the lumen is patent.

Seal the shaft into the tip of a 1 c.c. tuberculin syringe, using DeKhotinsky or other strong, waterproof cement. Be sure the needle does not protrude into the syringe chamber.

Mount the sample syringe in a syringe stop, and adjust the volume to about 1 c.c.

Fit the syringe into the cap of a 2 oz. dropper bottle, the standard type used by pharmacies to dispense nose drops, after first cutting off the top of the bulb and removing the glass tip. The revised dropper bulb becomes a bushing that holds the syringe in the plastic cap.

Fill the bottle with distilled water.

If the bedside tests are integrated, one sample syringe may be used for the bicarbonate and either the chloride or the total base test.

Acid Syringe.—

Following the instructions for the sample syringe, alter a second 1 c.c. tuberculin syringe. Set the volume at 0.7 c.c.

Fill the bottle with standard acid.

1 c.c. Tuberculin Syringe for Sodium Hydroxide Titration.—

Modify as follows: Rapidly rotate the tip in a Bunsen burner flame. As the glass softens, the orifice will slowly close. Allow this to proceed until the shrunken orifice will just admit the wire which fits in a 23 to 24 gauge needle, approximately 38 gauge wire.

The titrating syringe should also be fitted with the standard metal clip that steadies the plunger. This syringe may also be fitted into the cap of a 2 oz. dropper bottle.

Fill the bottle with standard base.

2 or More Titrating Test Tubes.—

These should be about 1 inch in outside diameter and 4 inches long (Pyrex rimless culture tube No. 9820).

Color Standard.—

Heat the neck of a titrating test tube and pull it out to form a narrow constriction that may be sealed later to form an ampule.

Place 2.5 c.c. of pH 7.4 buffer, made from *p*-Hydrone capsules, in the ampule.

Add 0.25 c.c. of 0.04% phenol red indicator.

Seal the ampule and place it in boiling water for 30 minutes.

While this sealed color standard will keep for an indefinite period, it should be checked about once a year by comparing it with a fresh standard made in a regular titrating test tube.

3 Test Tubes or Other Vessels to Hold the Color Standard and Titrating Test Tubes.—**1 2-oz. Bottle to Contain Standard Carbonate Reagent.—****1 2-oz. Dropper Bottle for Phenol Red Indicator.—**

The bottles and test tube holders can be mounted in a box or taped together to form a test kit.

Technic.—

Obtain 3 c.c. or more of blood by venipuncture in the usual manner.

Place in a clean dry tube of a volume not much larger than that of the sample.

Stopper the tube and allow a clot to form.

After the clot retracts, the serum may be obtained directly or by centrifuging. Exposure to air should be minimal until the serum has been separated from the cells. Plasma may also be used.

Draw about 0.1 c.c. of the serum or plasma into the sample syringe.

With the syringe pointing upward, draw the plunger out all the way and return it to zero. This rinses the syringe with the sample and eliminates bubbles.

Fill the sample syringe with serum and deliver into the titrating test tube.

The volume setting on the sample syringe may be any volume near 1 c.c. as long as it is kept constant. This permits the sample syringe to be used for either the total base or chloride tests, which are standardized by altering the sample volume.

Fill the acid syringe with the correct volume of N/10 nitric acid, and deliver it into the titrating test tube. See section on standardization below.

Agitate the serum-acid mixture for not less than 2 minutes. It is best to use a rotary motion that washes the solution up on the sides of the tube but does not form bubbles. This is a most important step as inadequate swirling can cause a significant error. If speed is essential, the specimen can be swirled for only 30 seconds; however, this decreases the precision of the test from ± 0.5 to ± 1 meq./L.

Add exactly 5 drops of indicator.

Fill the alkali syringe with 0.7 c.c. of N/10 sodium hydroxide.

Place the syringe in the titrating test tube so that the tip can be washed with the sample.

Lower the plunger slowly, rotating it to obtain better control.

Swirl the solution constantly during the titration and keep the syringe tip continually washed in the solution. As the end point is approached, add the alkali even more slowly to avoid momentary persistence of red color. Even though the color may fade again, do not go beyond the end point. If the titration is carried out properly with sufficient agitation, the end point will be gradually approached and almost no fading will occur.

The end point is determined by comparing the sample color with that of the color standard. The shade rather than the depth of color is noted. This can be seen clearly only by tilting the tube and color standard above but not against a white surface, and looking for the magenta hue or purplish cast in the thin layer of the liquid. An easy way to titrate, and at the same time to see the magenta hue, is to put a white towel in the lap, and, steadying the elbows against the sides of the body, hold the sample and color

standard above the towel. The magenta or purplish hue is seen best in daylight or bluish or artificial light. This hue comes up suddenly with the addition of not more than 0.005 c.c. of base.

To be sure the end point has been reached, note the reading on the syringe and then add 0.005 c.c. ($\frac{1}{2}$ division) more base. This should make the sample definitely more magenta than the color standard. If it does not, continue the titration. The beginner will tend to undertitrate.

Calculation.—

The volume of sodium hydroxide left in the syringe, expressed in hundredths of a c.c., equals the serum bicarbonate in meq./L.

Multiply the figure by 2.23 to obtain the result in volumes per cent.

Example.—

0.24 c.c. of alkali left in the syringe at the end point.

Serum bicarbonate level is 24 meq./L., or 53 vol. %. ($24 \times 2.23 = 53.52$.)

Standardization of the Method.—

Perform the bicarbonate determination as directed, using the standard carbonate solution as the sample and filling the acid syringe to the 0.7 c.c. mark. If the result obtained is 25 meq./L., the method is standardized. If not, adjust the set screw on the syringe stop of the acid syringe up or down and repeat the test until a result of 25 meq./L. is obtained on the standard solution. For example, if the end point were reached at 24 meq./L. (1 meq./L. low), it would be necessary to decrease the volume of acid.

Because the standard carbonate solution is unbuffered, the end point during the standardization titration is sharp and accurate, but there is more fading than with serum. If the red color lasts more than 30 seconds, the end point has been reached.

Standardization of the bicarbonate method in this manner tends to compensate for errors in standardizing the acid and alkali, and errors in the calibration of the syringes.

The set should be restandardized frequently, and whenever new reagents or syringes are used. It is a simple matter to run the standard along with the unknown. If an incorrect result is obtained on the standard, the result on the unknown may be adjusted accordingly.

This method is sufficiently accurate to replace the volumetric method of Van Slyke for routine clinical laboratory work.

BLOOD KETONES

A simple estimation of blood ketones in diabetic acidosis was suggested by Dumm and Shipley.¹ They alluded to the necessity of an early diagnosis and prompt treatment of diabetic acidosis. It is true that a high blood sugar level is not diagnostic of acidosis and a reliable determination of CO_2 combining power is not always obtainable in an emergency. Because there is a parallel between the blood ketone level and the severity of diabetic acidosis, a simple clinical method for the estimation of blood ketones is of distinct value. This method takes advantage of the suggested use of a dry powder for the qualitative detection of these compounds in the urine.²⁻⁴ The powder suggested has the following formula:

Sodium nitroprusside, 1 gm. (very finely ground).

Ammonium sulphate, 20 gm.

Anhydrous sodium carbonate, 20 gm.

¹Dumm, R. M., and Shipley, R. A.: *J. Lab. & Clin. Med.* **31**: 10, 1162-1163, Oct., 1946.

²Laughlen, G. F.: *Canad. J. M. Technol.* **5**: 3, 1943.

³Ingram, John: *Brit. M. J.* **1**: 512, 1944.

⁴Rabinowitch, I. M.: *Canad. M. A. J.* **52**: 602, 1945.

The three ingredients are mixed completely but are not ground together. The compound should be kept dry at all times. Stability has been shown to be complete for over one year.

The test is performed by placing a small pinch of the powder, 5 mm. in diameter, on a white filter paper. One drop of urine or serum gives a prompt violet color reaction which is absorbed into the paper and which may last for several hours.

The previous use of powder of this type has been suggested for the establishment of the presence of acetone and diacetic acid in urine, but Dumm and Shipley have shown that it gives equally good results in the determination of blood ketones. The same color reaction appears when serum of a high ketone content is added to the powder. The minimal blood level of total acetone bodies giving a definitely positive test is approximately 10 mg. per 100 c.c. as determined by the method of Shipley and Long.⁵ Thus, the test can quickly be made quantitative by the successive dilution of serum with distilled water.

The last dilution to give a positive reaction, when multiplied by ten, will give the blood ketone level within an accuracy of plus or minus 10 mg. per 100 c.c. For example, if the undiluted serum has been shown to give a positive test, then 1 c.c. is mixed with an equal amount of water. If a positive reaction is still present, additional portions of water are added until the test becomes negative. In a given sample, the last positive dilution might contain 1 c.c. of serum and 6 c.c. of water. This would represent a dilution of 1 part in 7, and the result would be approximately 70 mg. per 100 c.c.

This test was used by Dumm and Shipley in the diagnosis and treatment of diabetic coma. It was shown that it was a method by which one could easily follow the blood ketone levels during active therapy in diabetic acidosis. By virtue of its rapidity and simplicity, it has proved more valuable than the emergency determinations of CO_2 combining power in providing immediate laboratory confirmation.

PROTEIN-BOUND IODINE

Since the advent of the protein-bound iodine determination, physicians have become keenly aware of this test as a direct method for the measurement of thyroid activity. From 1941 on, as the interest increased, modifications of this determination have been made to the extent that at the present time it has become one of the better diagnostic tests.

In borderline cases of hypothyroidism and hyperthyroidism, the clinician frequently finds it exceedingly difficult to establish a diagnosis. In addition to these borderline cases, many patients with hyper- or hypometabolic manifestations present themselves for equally difficult differentiation between thyroid abnormality and other pathologic situations. It is therefore of utmost importance that there be at the physician's finger tips an accurate measurement of thyroid function.

Serum cholesterol and basal metabolic rate determinations have in the past been the only routine aids to the thyroid picture. Unfortunately, both the cholesterol and basal metabolic rate methods are influenced by a wide variety of physiologic and pathologic situations other than the thyroid. Therefore, their use is of a limited nature.

⁵Shipley, R. A., and Long, C. N. H.: *Biochem. J.* 32: 2242, 1938.

The vast amount of work done recently on the measure of thyroid function by the more direct method of the determination of the serum protein-bound iodine has, as stated above, caused this test to be more widely used. The chemical determination of protein-bound iodine is technically difficult. However, these technics in the hands of skilled personnel and performed in the correct laboratory environment are quite accurate and represent a real diagnostic tool. The methods, however, are not applicable to routine determinations of protein-bound iodine in the usual clinical laboratory.

It has been well shown that serum protein-bound iodine is representative of circulating thyroid hormone, and analysis of this compound should be a true index of thyroxine output.

Fundamentally, the procedure entails the precipitation of the serum protein, consequent ashing, and the protein-liberated iodide catalyzing a reduction between ceric and arsenite ions.

In children with mild hypothyroidism, before frank myxedema has developed, the finding of a low protein-bound iodine has proved of considerable value in establishing the need for thyroid therapy. The application of the test is particularly useful in young individuals in whom the need for treatment is more urgent and the difficulty in getting a true basal metabolic rate is apparent.

In cretinism, the protein-bound iodine determination is more reliable than the serum cholesterol. In children diagnosed as cretins, the protein-bound iodine values have been found to be very low (around 2 micrograms per cent), while a large number of noncretin children have shown values within the normal adult range of 4 to 8 micrograms per cent, according to the method used.

Classification of ophthalmic cases, as Graves' disease without hyperthyroidism, is aided by the normal protein-bound iodine values and the additional clinical evidence. Combined results of Salter and associates seem to indicate that the protein-bound iodine can be used to estimate the physiologic thyroid activity regardless of the structural status of the thyroid gland.

Advantages Over Basal Metabolic Rate Determinations.—Protein-bound iodine determinations are not affected by certain conditions which make almost impossible a true basal metabolic rate evaluation. These conditions are chorea, Parkinson's syndrome, moronism, mongolism, essential hypertension, cardiac failure, carcinoma, leukemia, or psychic disturbances. Other conditions which will affect the basal metabolic rate and not the protein-bound iodine value are Addison's disease, Simmonds' disease, anorexia nervosa, narcolepsy, exhaustion, sexual neurosis, prepuberal hypogonadism, etc.

Salter and co-workers also have shown that the protein-bound iodine values can be correlated better than the basal metabolic rate with clinical diagnosis. It has been found that out of 100 carefully selected cases, the basal metabolic rate gave 71 compatible and 29 incompatible reports, while the protein-bound iodine values gave 98 compatible and 2 incompatible reports as compared with clinical diagnosis.

Precautions.—Precautions must be taken against contamination, especially with iodine or mercury. Since elemental iodine is so volatile and in such prevalent use as an antiseptic, it is the greatest hazard. Tincture of

iodine must be avoided as an antiseptic on the skin of the patient before the sample is drawn. The absence of any tincture of iodine must also be ascertained on any persons carrying out the actual determination. If elemental iodine is being used in any chemical determination or histologic procedure (i.e., Nessler's solution or Gram staining) in the same room, irregular and unpredictable contamination is almost inevitable. It is therefore best to devote an entire small room to the iodine work if accurate results are to be obtained.

In direct contrast to this difficulty, which is usually avoidable, once the patient has been administered any form of organic iodine, such as Lipiodol, Priodax, Skiodan, Diodrast, or tetraiodophenolphthalein, used in roentgenologic visualization, the binding of the iodine compound onto the serum protein will invalidate the determination. This contamination may remain effective up to 18 months.

It is not necessary for the patient to be in a fasting state; neither is it necessary to deny inorganic iodine, such as the type used in dietary therapy (Lugol's, kelp, iodized salt, etc.), for all the inorganic iodine is removed from the protein precipitate early in the assay. This is an obvious advantage in that prognosis can be accurately charted during therapy.

Normal Range.—The normal range for the protein-bound iodine is from 4 to 8 micrograms per cent for the dry ashing procedure. Normal range for acid digestion method, which is somewhat new, has been tentatively accepted as between 4 and 8 μ g. Abnormally low results are found in hypothyroidism, myxedema, and cretinism, and slight decreases are found in hypoproteinemia or malnutrition. High results are found in hyperthyroidism, and in pregnancy as early as the third week.

Specimen Collection.—Four cubic centimeters of serum or plasma are desirable. The syringe and barrel must be chemically clean and free of contaminants. Absolutely no tincture of iodine should be used in the venipuncture. Hemolysis will definitely interfere with the results and therefore the serum should be removed at the earliest possible moment.

Patient's History.—The patient must not have had Priodax, Lipiodol, Skiodan, Diodrast, tetraiodophenolphthalein, barium (used in gastrointestinal series), radioactive iodine, or mercurial diuretics. If any of these has been administered, there must be a resting period before accurate determinations can be made. In the case of the organic dyes, 12 to 18 months may be required before the determination can be accurately made. In the case of the other compounds, a resting period of at least one month must be permitted.

Method I. Protein-Bound Iodine Determination*

Method of Choice

Equipment.—

Clean glassware, washed with nitric acid once, tap water 3 or 4 times, distilled water 3 or 4 times, and once with either doubly distilled water or acid boiled water.

150 by 15 mm. Pyrex test tubes, approximately 20 c.c. These are used for precipitation and washing of protein.

180 c.c. electrolytic beakers, used for digestion and oxidation.

*Procedure used by Fifth Army Area Medical Laboratory. Personal communication from Major M. C. Hutchinson.

Digestion and oxidation are performed on a Lindberg hot plate at $150^{\circ}\text{C.} \pm 10^{\circ}\text{C.}$ under a fume hood. The temperature is controlled by laying a thermometer, its bulb covered with sand, on the back surface of the hot plate.

Reagents.—

Doubly Distilled Water or Acid Boiled Water.—

Doubly distilled water is the second distillation from dilute sodium hydroxide.

Acid boiled water is 2,000 c.c. of singly distilled water plus 0.5 c.c. of sulphuric acid.

Boil off approximately 300 c.c.

This distilled water is used for the final washing of all glassware, in preparing solutions, and in diluting the results of digestion.

Sulphuric Acid.—

This must previously have been boiled and cooled before use.

10% Perchloric Acid.—

This is used as the protein precipitant. It is made by using 10 gm. per 100 c.c.

Chromic Acid.—

Dilute 3.03 gm. of CrO_3 to 1 liter with distilled water; 0.5% sodium chromate may be used.

Combined Arsenious Acid (0.2 N), Sulphuric Acid, and Sodium Chloride.—

Dissolve 4.9 gm. As_2O_3

3.5 gm. NaOH

in 50. c.c. distilled water.

Dilute to 200 c.c. with distilled water.

Neutralize to phenolphthalein indicator with concentrated sulphuric acid.

Add 55.8 c.c. of concentrated sulphuric acid.

Dilute to 500 c.c. with distilled water.

Test the reagent here as in *a* below. If it is satisfactory,

add 2.5 gm. NaCl to 100 c.c. of the arsenious acid.

Test as in *b* below.

If this test is satisfactory,

add 2.5 gm. to each 100 c.c. of the arsenious acid-sulphuric acid reagent.

Ceric Ammonium Sulphate (0.02 N).—

Dissolve 12.665 gm. of ceric ammonium sulphate $(\text{NH}_4)_2\text{Ce}(\text{SO}_4)_2 \cdot 2\text{H}_2\text{O}$

and 52 c.c. of concentrated sulphuric acid

in 700 c.c. of distilled water.

Let stand overnight, and then dilute to 1 liter.

The solution is 1.9 N in sulphuric acid.

Chloric Acid Reagent (28%).—

Dissolve 500 gm. of KClO_3 with heat

in 900 c.c. of distilled water. While hot,

add 400 c.c. of 70 to 72% perchloric acid slowly with stirring.

Store in a freezing compartment for 24 hours.

Filter while cold.

Store the chloric acid (supernatant fluid) in a cool dark place. A freezer is preferable but the freezing compartment of a refrigerator is satisfactory.

Standard Iodate Solution.—

Dissolve 168.5 mg. KIO_3 , which has been dried in a vacuum desiccator, in distilled water and dilute to 1 liter with distilled water. This stock solution contains 100 μg per c.c.

Working Solution of Iodate.—

The working solution is prepared fresh each week.

Dilute 1 c.c. of stock to 1 liter with distilled water.

This solution contains 0.10 μg per c.c.

Standard Iodate Solution.—

This solution is used as the standard in each run:

Dilute 6 c.c. of the working solution

with 10 c.c. of distilled water.

This solution contains 0.0375 gamma per c.c.

Reagent Contamination Tests.—

a. Arsenious Acid-Sulphuric Acid-Without Sodium Chloride, Ceric Solution, and Distilled Water: Test as follows:

REAGENT	TUBE 1	TUBE 2	BLANK
Arsenious (0.2 N) - H ₂ SO ₄ reagent without NaCl ----	2 c.c.	1 c.c.	0 c.c.
Distilled water -----	4 c.c.	5 c.c.	7 c.c.
Ceric sulphate (0.02 N) -----	1 c.c.	1 c.c.	0 c.c.

Equal loss of color in tubes 1 and 2 indicates ceric contamination. Greater loss of color in tube 1 indicates arsenious acid-H₂SO₄ contamination. Moderately greater loss of color in tube 2 shows water contamination. Satisfactory if fading is less than 2% in 60 minutes.

b. Chromic Acid and Sodium Chloride Reagent Test:

REAGENT	TUBE 1	TUBE 2	BLANK
Chromic acid -----	0.4 c.c.	0 c.c.	0 c.c.
Arsenious-NaCl-H ₂ SO ₄ -----	2.0 c.c.	2 c.c.	0 c.c.
H ₂ O -----	3.6 c.c.	4 c.c.	7 c.c.
Ceric -----	1.0 c.c.	1 c.c.	0 c.c.

If tube 1 fades more than 2% greater than tube 2 in 60 minutes, it indicates chromic acid contamination. If tubes 1 and 2 show more than 10% transmission fading, the reagents are unsatisfactory and it will be necessary to decrease the sodium chloride in the arsenious acid reagent (iodine in the NaCl).

All the above tests are read in a Coleman Universal spectrophotometer at 420 mμ with water as a blank.

Patient's Serum.—

Fasting samples are required. If any type of iodine therapy has been administered, there should be a lapse of at least seven days between medication and venipuncture. The iodine compounds used for bronchiography, myelography, etc., may cause false high values for a year or longer.

Syringe and tubes should be acid cleaned and thoroughly rinsed.

Blood should not be drawn with iodine in the vicinity of the patient.

Serum, at least 3 c.c., must be separated as soon as possible, and should be mailed with a clinical abstract covering diagnosis, iodine medication, etc.

Technic.—

a. Precipitation of Protein.—

Measure 1.5 c.c. of serum into a 150 by 15 mm. Pyrex test tube.

Add rapidly (blow in) 15 c.c. of perchloric acid solution.

Allow to stand 10 minutes, then centrifuge 10 minutes at 2,000 r.p.m. and decant.

Again add 15 c.c. of perchloric acid solution to the precipitated protein, resuspend, and centrifuge again at 2,000 r.p.m. for 10 minutes. Discard the supernatant fluid.

b. Digestion—Oxidation.—

With the aid of three 5 c.c. portions of chloric acid, transfer the precipitated protein to a 180 c.c. electrolytic beaker (total 15 c.c. chloric acid).

Add 1 c.c. of chromic acid solution.

Add 10 to 15 glass beads to each beaker.

Evaporate on a hot plate with a surface temperature of 150° C. ± 10° C.

After crackling, caused by decomposition of chloric acid, ceases and white fumes appear, add 0.5 c.c. of chloric acid to each beaker.

Thereafter, add 1 or 2 drops of chloric acid from time to time to maintain chromium in its orange hexavalent state.

CAUTION: Reduction of chromium to the green trivalent state for more than a few seconds is associated with loss of iodine.

Evaporate to an estimated volume of 0.3 to 0.5 c.c.
Iodine may be lost if complete dryness is obtained. With practice, the visual estimation of the desired volume can be made with negligible error.

Set up a reference beaker with glass beads and 0.5 c.c. of solution.
Include 6 reagent blanks in each run, 4 to be used later as standards, 2 as blanks.

c. Spectrophotometry.—
Allow beakers to cool, then add 10 c.c. of distilled water to all unknowns and 2 blanks.
Prepare standards by adding the following to the remaining 4 blank beakers:

REAGENTS	s-1	s-2	s-3	s-4
0.0375 mcg/c.c. Standard	1 c.c.	2 c.c.	3 c.c.	4 c.c.
Distilled water	9 c.c.	8 c.c.	7 c.c.	6 c.c.
Mcg iodine in 4 c.c. aliquot	0.015	0.03	0.045	0.06
Using 1.5 c.c. serum and 4 c.c. aliquot				
Equivalent mcg/100 c.c. serum	2.5	5.0	7.5	10.0

Warm all the beakers gently on the hot plate to effect solution.
Pipette 4.0 c.c. aliquots of the contents of each beaker into 105 by 19 mm. cuvettes.
Add 2.0 c.c. of combined arsenious acid (0.2 N)-sulphuric acid-sodium chloride solution.

Mix and allow to stand at room temperature for 30 minutes, or place in a water bath at room temperature for 15 minutes.

Add accurately to each tube 1 c.c. of 0.02 N ceric ammonium sulphate solution with a delay of ½ minute between additions to successive tubes in the series.

Read percentage transmission in a Coleman Universal spectrophotometer at 420 mμ against a water blank.

Each tube should be read ½ minute after adding the ceric solution.
With samples having a high iodine content, as shown by rapid decolorization of the ceric, a 1 c.c. aliquot of the beaker contents is taken, and 3 c.c. of blank contents added, and the tube treated as above, beginning with the addition of 2 c.c. of combined arsenious acid-sulphuric acid-sodium chloride solution.

Calculations.—
Multiply the results obtained from the graph (Fig. 132) by 4 to obtain the gammas of iodine per 100 c.c. of serum.

Subsequent Readings and Calculations.—
The time of subsequent readings depends on the number of unknowns being run. No more than 10 unknowns should be run at one time. The calculations and readings are best described by showing an actual run.

TABLE 39

TUBE NO.	½ MIN. READING	8½ MIN. READING	16½ MIN. READING	24½ MIN. READING	32½ MIN. READING	40½ MIN. READING
1	16.5	26.0	42.0	58.5	71.0	79.0
2	16.5	21.5	28.5	37.5	48.0	57.5
3	16.5	22.0	29.5	39.0	50.0	60.0
4	16.3	23.5	35.0	50.0	62.0	71.0
5	16.3	21.0	27.5	37.0	47.0	56.0
6	16.3	20.0	25.0	32.0	40.0	48.0
7	16.5	21.0	28.5	37.5	48.0	57.5
8	16.3	20.5	26.0	33.0	41.5	50.0
9*	16.5	21.5	29.0	39.0	50.0	59.5
10	16.5	18.5	22.0	26.0	30.0	35.5
11	16.5	20.0	25.0	31.0	38.0	45.0
12*	16.5	21.5	30.0	40.0	51.0	60.0
13	16.5	23.0	34.0	48.0	61.0	69.0
14	16.5	18.5	20.0	22.0	25.0	27.0
15	16.3	18.0	20.0	22.0	25.0	27.0

*See text.

Samples 1 through 8 were unknown sera. Sample 9 was the same as standard 12 except that iodate was added to the beaker and digested the same as the sera. Samples 10, 11, 12 and 13 were the four standards equivalent to 2.5, 5.0, 7.5, and 10 gammas of iodine. Samples 14 and 15 were the two blanks. The per cent transmittance readings are given in Table 39.

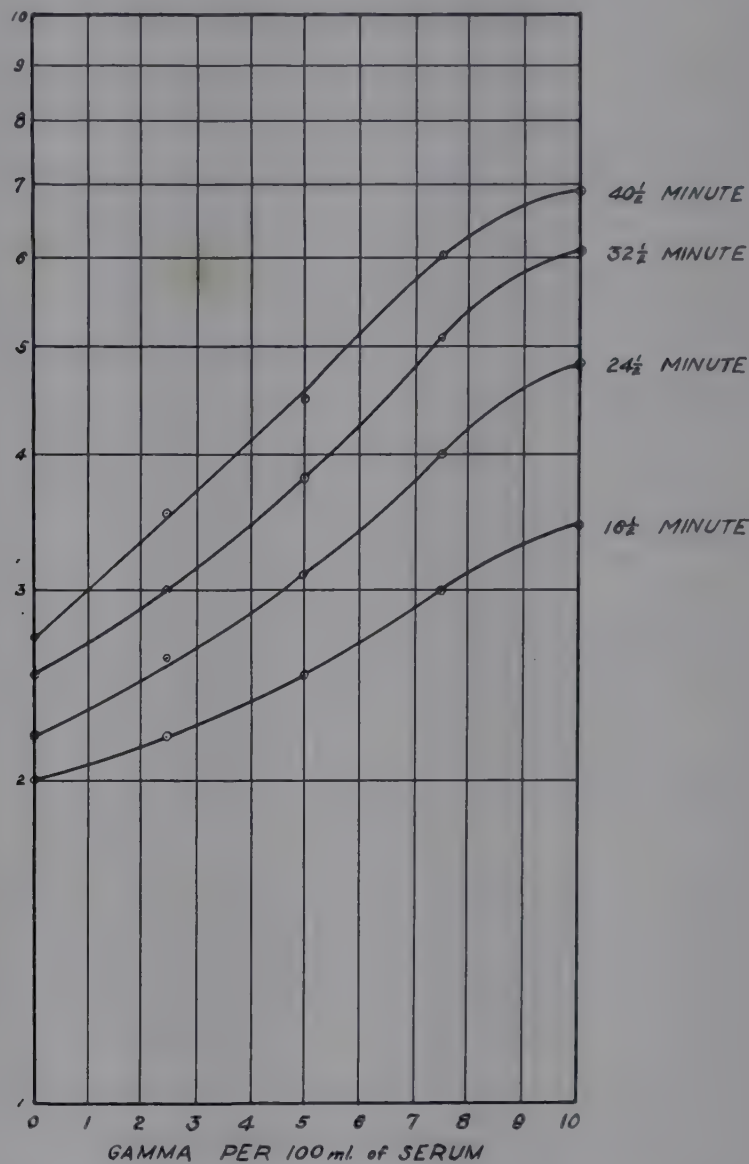


Fig. 132.—Graph for plotting bound iodine.

Results calculated from the graph (Fig. 132) are given in Table 40.

TABLE 40

TUBE NO.	1	2	3	4	5	6	7	8
16½ min. (gamma iodine per 100 c.c. of serum)	Too high to read	6.8	7.3	10.5	6.2	5.0	6.8	5.3
24½ min. (gamma iodine per 100 c.c. of serum)	Too high to read	6.8	7.3	10.5	6.6	5.2	6.8	5.5
32½ min. (gamma iodine per 100 c.c. of serum)	Too high to read	6.9	7.3	10.5	6.8	5.3	6.9	5.5
40½ min. (gamma iodine per 100 c.c. of serum)	Too high to read	7.1	7.5	10.7	6.8	5.4	7.1	5.8
Average (gamma iodine per 100 c.c. of serum)		6.9	7.3	10.5	6.6	5.2	6.9	5.5

Tube No. 1 read too high. Therefore, 1 c.c. of the unknown and 3 c.c. of the blank were used and the readings repeated. The 16½ minute reading was 26.5. The 24½ minute reading was 34. The result determined from the graph was multiplied by 4 and the amount reported was 24 gammas per 100 c.c. of serum.

Interpretations.—**Micrograms per 100 c.c. of serum:**

0 to 3	Hypothyroid
3 to 4	Hypothyroid (questionable)
4 to 9	Euthyroid
9 to 10	Hyperthyroid (questionable)
10 up	Hyperthyroid.

Comments.—

a. The principal source of difficulties is with the reagents. Some chemicals were found unsatisfactory. Mallinckrodt's analytical reagent grade chemicals are excellent. Ceric ammonium sulphate is purchased from G. Frederick Smith Chemical Company, Columbus, Ohio. Using these chemicals, few reagent difficulties have appeared.

b. The digestion temperature has been found to be critical. Leffler speaks of allowing the tubes to boil for 1 hour. In this laboratory, boiling resulted in loss of iodine. The browning of paper as suggested by Leffler to obtain a certain temperature would seem to be a very variable temperature control. Furthermore, even with a Lindberg hot plate the control has to be changed occasionally to hold the temperature at $150^{\circ} \pm 10^{\circ}$ C. At this temperature the solution does not come to a true boil and recoveries of digested iodate compared with undigested iodate have been excellent. (See tube 9, Table 39, iodate as compared with tube 12 undigested iodate.) Digesting at 150° C. usually requires 2 hours or less.

It should be noted that if the serum iodine is high and a smaller aliquot of the digested sample is to be used, then the dilution to 4 c.c. should be made with the blank solution and not with water.

The half-minute reading of all tubes is made as a check on the pipetting. The variance in the half-minute readings should not be greater than 0.5% transmission.

Leffler makes his calculations from the 20-minute readings. We have found it more accurate to make the calculations from 3 or 4 graphs as described and to average the results. This procedure of calculating has improved the recovery and appears desirable.

It is estimated that a well-trained technician could perform 20 determinations per day. This estimate includes the cleaning of glassware and the preparation of reagents. The spectrophotometric readings require two people, one to perform the readings and the other to pipette the ceric solution (pipetting requires 10 minutes or less).

The combined arsenious acid reagent deteriorates slowly. It is usually satisfactory for 2 months stored at room temperature. If there is little or no decolorization of the ceric solution, or the difference between the standards is insufficient, it may be due to the arsenious acid reagent. A freshly prepared reagent will usually correct this difficulty.

Chloric Acid Reagent.—One reagent difficulty was traced to a new chloric acid reagent prepared using an old bottle of perchloric acid. There was practically no decolorization of the ceric solution either in the undigested iodine standards or the unknowns. It is likely that the acid had weakened in long storage, thus leaving excess potassium chlorate (known inhibitor) when the chloric acid was prepared.

Textbooks state that chloric acid decomposes at 40° C., yet Zak, Leffler, and others give directions for preparing the chloric acid digestion reagent at temperatures in excess of 40° C. Is the oxidizing digestion reagent chloric acid? This is a theoretical question because regardless of the exact chemical composition, an adequate oxidizing digestion solution results in most instances by their procedure.

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4. Leffler, H. H.: *Am. J. Clin. Path.* **24**: 483, 1954.
5. Zak, B., and others: *Am. J. Clin. Path.* **23**: 603, 1953.
6. Modifications by Fifth Army Area Medical Laboratory.

Technic of Determination of Protein-Bound Iodine Method of Barker, Humphrey, and Scoley¹

This is a modification of an alkaline-incineration method² which is practicable for a clinical laboratory. Extreme care must be used in the quantitative measurement of 0.05 μg protein-bound iodine per cubic centimeter of plasma as well as in avoiding contamination.

Reagents.—

Make all solutions in doubly distilled water. If the amount of iodine in the water is still too high to give a satisfactory blank, it may be necessary to carry out these distillations with such reagents as alkali and permanganate.

All reagents used should be of the highest purity obtainable. However, the iodine content in each instance must be judged from the blank values rather than from estimates shown on the labels. It is often necessary to turn to a new source of supply or to a different lot number in case of contamination.

Zinc Sulphate Solution.—

Dissolve 100 gm. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in distilled water in a liter volumetric flask and dilute to 1 liter with distilled water.

0.5 N Sodium Hydroxide Solution.—

Dissolve 20 gm. of sodium hydroxide in distilled water in a liter volumetric flask and dilute to 1 liter with distilled water. Titrate against the zinc sulphate solution as follows:

Dilute 10 c.c. of the zinc sulphate solution with 50 to 70 c.c. of distilled water. Titrate with the sodium hydroxide solution and adjust until 10.8 to 11.2 c.c. of the 0.5 N sodium hydroxide solution will be required to produce a faint permanent pink color with phenolphthalein indicator.

2 N Hydrochloric Acid Solution.—

Add 200 c.c. of concentrated hydrochloric acid, c.p., to distilled water in a liter volumetric flask, and dilute to 1 liter with distilled water.

7 N Sulphuric Acid.—

Add 196 c.c. of concentrated sulphuric acid, c.p., to distilled water in a liter volumetric flask. Cool and dilute to 1 liter with distilled water.

Stock Standard Iodide Solution.—

Use either NaI or KI of the highest purity desiccator-dried chemical. If sodium iodide is chosen, dissolve 118.1 mg. in distilled water in a liter volumetric flask and dilute to 1 liter with distilled water.

If potassium iodide is used, dissolve 130.8 mg. in distilled water in a liter volumetric flask and dilute to 1 liter with distilled water.

The concentrated stock solutions contain 100 μg iodine (I) per c.c.

Dilute Stock Iodide Standard.—

Add 2 c.c. of the stock standard iodine solution to distilled water in a liter volumetric flask and dilute to 1 liter with distilled water.

Standard Iodide Solution (0.04 μg iodine per c.c.).—

This is the solution used in each determination.

Dilute 10 c.c. of the dilute stock iodide standard to 50 c.c. with distilled water.

All of these iodide solutions have been found to keep indefinitely at refrigerator temperatures, but it is preferable to prepare the most dilute standard fresh every week or two.

0.02 N Ceric Ammonium Sulphate Solution.—Dissolve 12.65 gm. of ceric ammonium sulphate in 500 c.c. of distilled water containing an added 230 c.c. of 7 N sulphuric acid. When the solution is clear, dilute to 1 liter with distilled water.

¹Barker, S. B., Humphrey, M. J., and Scoley, M. H.: *J. Clin. Invest.* 30: 55, 1951.

²Salter, W. T., and McKay, E. A.: *Endocrinology* 35: 380, 1944.

0.1 N Sodium Arsenite Solution.—

Dissolve 4.95 gm. of As_2O_3 in 25 c.c. of 4% (N/1) sodium hydroxide, warming to hasten solution.

Dilute with about 300 c.c. of distilled water.

Add dilute sulphuric acid until the solution is slightly acid to litmus paper. If the 7 N acid is used, about 4 c.c. will be required.

Dilute to 1 liter with distilled water.

If sodium arsenite itself is used, dissolve 6.5 gm. of NaAsO_2 in distilled water and dilute to 1 liter with distilled water. Do not use any acid or alkali with this salt.

Analytical Procedure.—

There are four principal steps involved: (1) precipitation and washing of plasma proteins, (2) drying and incineration of sample, (3) dissolving iodide from the ash, and (4) colorimetric determination of iodide present.

(1) Precipitation³ and Washing of Plasma Proteins.—

Centrifuge about 10 c.c. of the blood sample and draw off the serum or plasma.

Pipette duplicate aliquots of 1 c.c. each into 15 by 125 mm. Pyrex test tubes.

Dilute with 7 c.c. of distilled water.

Add 1 c.c. of 10% zinc sulphate solution.

Mix the contents of each tube with a glass stirring rod about 2 mm. in diameter.

Add 1 c.c. of 0.5 N sodium hydroxide solution.

Thoroughly mix the solutions to insure even distribution of the alkali and complete precipitation of the proteins. Any material adhering to the rod can be removed by rubbing it with a rotary movement against the inside wall of the tube.

Centrifuge the tubes for 10 minutes to pack the precipitate.

Pour off the supernatant fluid.

Add 10 c.c. of distilled water.

Resuspend the protein by means of the same stirring rod originally used. Stirring should be only sufficiently vigorous to distribute the zinc proteinate uniformly throughout the solution; if overzealously carried out, the precipitate may be so finely divided that it cannot be easily centrifuged down.

Centrifuge again and again discard the supernatant fluid.

Repeat the washing process twice more, making a total of three washings. The original precipitation is *not* included as a washing.

(2) Drying and Incineration.—

After the last supernatant solution has been poured off, add 0.8 c.c. of 4 N sodium carbonate solution.

Thoroughly stir into the precipitate with the same stirring rod used in the washings. Removal of material clinging to the rod can be accomplished by rubbing as described previously, followed by 0.2 c.c. of the 4 N sodium carbonate solution added dropwise down the rod. This last portion of alkali can easily be mixed with the sludge already present without use of the stirring rod.

Place the tubes in an oven set at 85° to 95° C. to drive off the water. This usually requires 12 to 18 hours. Carry out overnight.

After the alkali and zinc proteinate have been thoroughly dried, carry out the ashing by placing the tubes in a muffle furnace for $2\frac{1}{2}$ hours at $600^\circ \pm 25^\circ$ C. At the end of that time, remove the tubes and allow to cool to room temperature.

(3) Dissolving Iodide From the Ash.—

Add 2 c.c. of 2 N hydrochloric acid with due caution to avoid excessive effervescence.

Use a glass rod to mix any unreacted ash with the acid.

Add 2 c.c. of 7 N sulphuric acid

and 3 c.c. of distilled water.

Stir until the reaction appears to be completed.

Transfer to a clean tube and centrifuge briefly to pack the insoluble material.

³Somogyi, M.: J. Biol. Chem. 86: 655, 1930.

(4) **Determination of Iodide.**⁴—

Use a pair of colorimeter tubes for each tube.

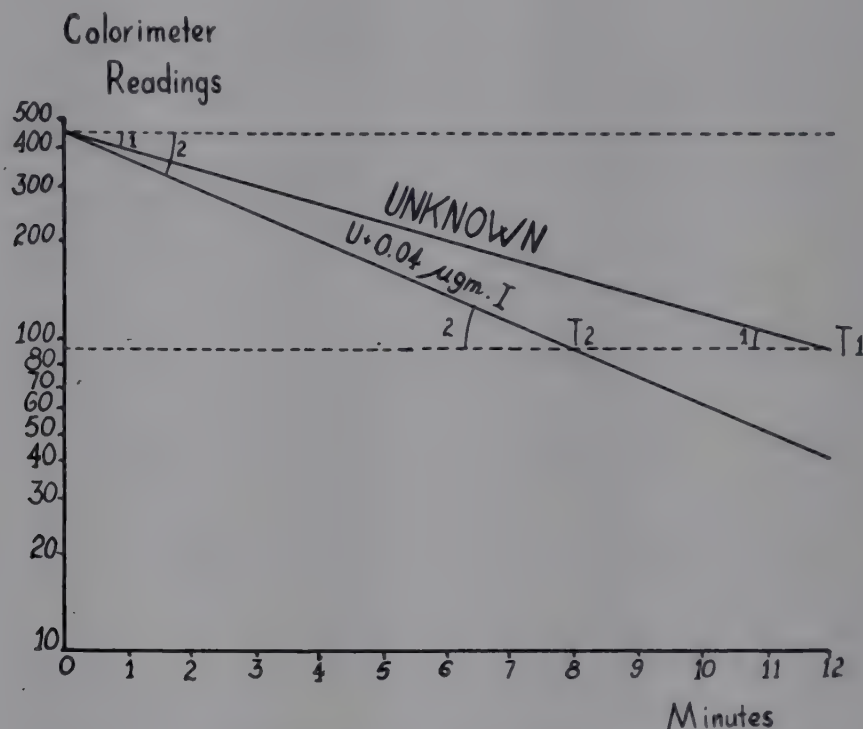
To one of the pair add 1 c.c. of distilled water containing 0.04 μg of iodine as sodium or potassium iodide.

To the other, add 1 c.c. of distilled water.

Pipette a 3 c.c. aliquot of the supernatant fluid in the sample tube into each colorimeter tube.

Add 0.5 c.c. of the sodium arsenite solution to each tube.

Mix by some technic which prevents both loss of solution and contamination.



$$\tan < 1 = \frac{\text{Initial colorimeter reading} - \text{final colorimeter reading}}{t_1}$$

This is an expression of the decolorization of the U sample. Since the assumption is being made that the decolorization is due entirely to I,

$$\frac{\text{Difference in colorimeter readings}}{t_1} = U \mu\text{g I in sample}$$

$$\text{Difference in colorimeter readings} = U \times t_1$$

In similar fashion:

$$\tan < 2 = \frac{\text{Difference in colorimeter readings}}{t_2} = U + 0.04 \mu\text{g I}$$

$$\text{Difference in colorimeter readings} = (U + 0.04 \mu\text{g I}) t_2$$

Since the differences in colorimeter readings are identical,

$$U \times t_1 = (U + 0.04 \mu\text{g I}) t_2 = U t_2 + 0.04 \mu\text{g I} \times t_2$$

$$U t_1 - U t_2 = 0.04 \mu\text{g I} \times t_2$$

$$U = \frac{t_2 \times 0.04 \mu\text{g I}}{t_1 - t_2}$$

Fig. 133.—Method of plotting results for calculation of PBI.

Place the colorimeter tubes in a well-stirred constant temperature water bath maintained at $39^\circ \pm 0.1^\circ \text{C}$. for 10 minutes to come to temperature.

Warm the ceric ammonium sulphate solution in the bath.

Since the actual determination of iodide is accomplished by measuring iodide catalysis of the rate of decolorization of yellow ceric ammonium sulphate by arsenious acid, the times at which colorimeter readings are made must be definitely established.

1 c.c. of the ceric solution must be added to each tube with adequate time allowance for reading the color change in the previous tube. This can be accomplished by leaving a 30-second interval between tubes and by setting up a maximum of only 12 tubes in a single series.

⁴Sandell, E. B., and Kolthoff, I. M.: *Mikrochimica acta* 1: 9, 1937.

Make colorimeter readings at 6 and 12 minutes after adding the ceric sulphate solution to each tube.

If 30 seconds is not adequate for making the reading, more time must be allowed, and fewer determinations made in any one series. Since the measurement depends entirely upon the decrease in amount of color, the pipetting of the 1 c.c. of ceric solution must be highly accurate, even though done rapidly. Mixing must be rapid and thorough.

Use the blue filter No. 42 and plot the Klett-Summerson readings on semilog paper against the time in minutes. Ordinary graph paper is suitable for instruments giving readings in terms of per cent light transmission.

The colorimeter setting at zero time is obtained for any given batch of reagents by quickly reading a reagent blank immediately after the addition of the ceric sulphate.

The lines describing the two reaction rates are drawn, using the points for 0, 6, and 12 minutes, as shown in Fig. 133. Then the horizontal lines indicating times required to reach equal amounts of decolorization are drawn.

The amount of iodine in the colorimeter tube without added iodide is calculated from the following equation, obtained from similar triangles (Fig. 133):

$$\mu\text{g I in colorimeter tube} = \frac{0.04 \times t_2}{t_1 - t_2}.$$

t_1 = time in minutes at intercept of the rate of decolorization in the tube containing no extra iodide with the designated horizontal.

t_2 = time in minutes at intercept of the rate of decolorization in the tube containing added iodide with the designated horizontal.

The reagents contain variable amounts of iodide; the total must be determined by blank analyses beginning with the zinc sulphate and sodium hydroxide solution, and carrying through the entire procedure, including the washings with 10 c.c. of distilled water, addition of 1 c.c. of sodium carbonate, drying, ashing, treatment with hydrochloric acid and sulphuric acid, and the final colorimetric assay on 3 c.c. aliquots just as with an unknown sample. The **blank value** calculated by the same formula shown previously has been 0.015 to 0.017 μg of I per tube in the laboratory of Barker and associates.

Since it is customary to express the values in terms of μg per 100 c.c. of plasma, the complete calculation would be as follows:

$$\text{Plasma protein-bound iodine } \mu\text{g I per 100 c.c.} = 100 \times \frac{7}{3} (\text{I in final aliquot} - \text{blank I})$$

where the factor of 7/3 has been introduced to correct for the 3 c.c. aliquot taken for colorimetric evaluation from the 7 c.c. of acid supernatant fluid from the ashed sample.

If so much iodide is present in the sample that decolorization is nearly complete in the second tube, with 0.04 μg of added I, the determination may be repeated using less than the 1 c.c. of plasma usually called for. Computation of the results must be adjusted as necessary to cover this change.

NOTES AND PRECAUTIONS:

Scrupulous care must be exercised to insure clean glassware. A detergent-soap mixture such as "Lakeseal"* is preferred for routine cleaning. Rinse this solution off with a rinse of tap water, followed by doubly distilled water.

In the precipitation and washing of plasma proteins, the blood sample may be collected with or without an anticoagulant. The advantages of using plasma are the speed with which it is obtained and the larger volume of fluid resulting; with serum, no foreign material need be added to the blood, which is an advantage. No difference has been found between the PBI values for serum and plasma. It is well to prepare bottles containing 0.1 c.c. of 20% potassium oxalate dried in an oven. The labels should be distinctive to avoid exchange with bottles from other laboratories. If serum is to be used, a paraffined tube is advantageous to allow easy clot retraction.

The procedure of washing the zinc proteinate precipitate by resuspension in distilled water and centrifugation has been found both by chemical and radioiodine tracer studies⁵

*Obtainable from the Finger Lakes Chemical Co., Etna, N. Y.

⁵The I^{131} used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

to remove between 99 and 99.5% of the inorganic iodine present. At plasma inorganic I levels of 2 $\mu\text{g } \%$ (for normal human beings) to 100 $\mu\text{g } \%$ (on some Lugol-treated patients), the residual amounts (about 0.01 to 1.0 $\mu\text{g } \%$) are not large enough to interfere. However, thyrotoxic patients being given 5 drops t.i.d. often are up to 300 $\mu\text{g } \%$, and one woman receiving 20 drops t.i.d. rose to 1,120 $\mu\text{g } \%$. Although it is of questionable therapeutic value to use such extremely high iodine doses, the situation occasionally is seen so that the laboratory must constantly be on the alert. If all iodine therapy is discontinued for at least four days prior to obtaining blood for protein-bound iodine levels, the plasma inorganic iodide will return nearly to the normal level, so that three washings are adequate. If the I^{131} uptake by the thyroid gland is also to be measured, an even longer period of time is required. This type of precaution is better than increasing the number of washings of the protein precipitate.

In the ashing process⁶ it is not necessary to take any elaborate precautions to insure absolutely even distribution of the protein-sodium carbonate suspension on the wall of each tube. However, it is desirable to avoid leaving the entire contents at the bottom of the tube, since this may lead to large bubble formation during drying. Often such a bubble will rise through the entire tube, carrying ahead of it considerable protein material and causing loss of protein-bound iodine.

The ashing time and temperature are not critical; variations of 2 or 3 hours and from 575° to 625° C. cause insignificant differences in results. At temperatures above 650° C., the Pyrex tube becomes soft and iodine also is lost. The ash in the recommended temperature range is gray rather than white, but the incineration is adequate to put all the organic iodine into extractable inorganic form. Below 500° C. there are so many products of incomplete ashing that the acid solution is yellow and substances are extracted which give a very high "iodine" value.

Use each tube three or four times and then discard to avoid excessive etching with possible loss of the sample.

When dissolving the iodide from the ash, the effervescence produced when the hydrochloric acid reacts with the ash is highly desirable for carrying off hydrogen sulphide formed from the sulphur-containing amino acids. If hydrogen sulphide remains when the ceric sulphate is added, insoluble ceric sulphide precipitates.

All steps in the colorimetric determination of iodide present must be carried out with great care to reproduce accurately all conditions of volumes, time, and temperature, since the measurement is made on a basis of iodide catalysis of a rate reaction. The actual conditions may be varied considerably, but those decided upon must be duplicated carefully from analysis to analysis if good agreement is to be had.

In this last phase, all mixing of solutions must be accomplished by some method which avoids both contamination and loss of solution, such as twirling each tube between the hands, flicking the bottom of each tube with several fingers in succession, or rapidly inverting each tube. The inversion or "flipping" procedure is preferred; this is carried out by grasping the colorimeter tube close to the open end between the thumb and first finger. The tube is inverted by raising it over the head and then is rapidly returned. With a little practice, one can achieve complete mixing of practically all miscible solutions in 8 to 10 such inversions without losing a drop. This method is especially valuable when the ceric sulphate solution must be mixed quickly and completely with the use of only one hand.

The method for calculation has been devised to rule out any possible effect of substances remaining in the final acid solution from the ash. Many nonvolatile compounds could conceivably be present in solution to alter the final colorimetric assay. Any such substance should affect the standard 0.04 μg of sodium iodide added to one aliquot as well as the unknown itself.

⁶For the most consistent results, it is desirable to have a muffle furnace available exclusively for use of the iodine laboratory. Random contaminations have proved a serious problem to Barker and co-workers when using one in the institution's water laboratory. No source for the added iodide was ever found.

A series of 57 comparisons was carried out on duplicate clinical plasma samples using this method and the distillation method described some time ago.⁷ There was no significant difference between the two series. In 43 plottings, with different persons plotting the same colorimeter readings, good agreement was obtained.

The usual method of calculating by preparing a curve of standards and interpreting colorimeter readings of unknown solutions from this curve is much simpler, but there is a possibility of the presence of some substance which would enhance the decolorization or interfere with it. The two methods of computation have been compared in a number of clinical samples; the standard curve technic was consistently higher, by 0.8 $\mu\text{g } \%$ using the 6-minute readings, and by 1.3 $\mu\text{g } \%$ using the 12-minute readings. Recovery of the added 0.04 μg of standard iodide was 99.3 and 100%, respectively. No basis for this difference can be offered at this time. It is obvious that use of the simpler form of calculation will necessitate upward revision of the values to be considered normal by about 1 $\mu\text{g } \%$.

The **normal range**, according to data obtained in Barker's research, appears to be 3.5 to 8 $\mu\text{g } \%$.

Protein-Bound Iodine, Method of Leffler⁸

The method of Leffler is a modification of the chloric acid-digestion method of Zak and his associates.⁹ The author expresses warnings in carrying out this method; namely, the contamination of glassware to be avoided, fear of explosion during preparation of chloric acid, loss of iodine during digestion, and failure of the chlorometric system to function. Since traces of iodine, constantly present in laboratory air, have great affinity for glass surfaces, all glassware should be kept in acid dichromate cleaning solution until just before use. It should then be thoroughly washed in hot tap water, rinsed in distilled water, and dried rapidly in the air. This leaves glassware free of contamination.

Reagents.—

Chemicals of the highest reagent grade with low iodine content are required. Once-distilled water has been found satisfactory.

Trichloroacetic Acid, 15% Solution.—

Dissolve 150 gm. of trichloroacetic acid in distilled water and dilute to 1 liter with distilled water.

Sodium Chromate, 0.5% Solution.—

Dissolve 5 gm. sodium chromate in distilled water and dilute to 1 liter with distilled water.

Stock Standard Iodate Solution.—

Dissolve 168.5 mg. of KIO_3 , previously dried in a desiccator, in distilled water in a liter volumetric flask, and dilute to 1 liter with distilled water. This gives an iodine concentration of 100 μg per c.c.

The working solution must be made fresh each week.

Iodate Working Standard.—

Each c.c. contains 0.1 μg of iodine.

Dilute 1 c.c. of the stock standard iodate solution to 1 liter in a liter volumetric flask.

Chloric Acid, Approximately 28% Solution.—

Since the question of explosion has caused some workers to fear this reagent, a detailed description of its preparation is given.

Place 200 gm. of crystalline potassium chlorate in a chemically clean Pyrex liter beaker.

Add 360 c.c. of distilled water.

⁷Barker, S. B.: Plasma Protein-Bound Iodine in the Diagnosis of Thyroid Disease, in Soskin, S.: Progress in Clinical Endocrinology, New York, 1950, Grune & Stratton, p. 61.

⁸Leffler, H. H.: Am. J. Clin. Path. 24: 483, 1954.

⁹Zak, B., Willard, H. H., Myers, G. B., and Boyle, A. J.: Anal. Chem. 24: 1345, 1952.

Place on an asbestos gauze mat over a Bunsen flame, and with occasional stirring, heat until dissolved (25 to 30 minutes).

When solution is complete, remove the beaker from the flame, and while still hot add slowly and intermittently, with constant stirring

166 c.c. of 70% perchloric acid.

The heat of reaction might cause violent boiling and spattering if the perchloric acid is added too rapidly, but no trouble should be experienced if added carefully a few c.c. at a time, stirring thoroughly between each addition.

When finished, potassium perchlorate will form a white precipitate and the solution will be light green-yellow from the liberated chlorine.

Cool for a few minutes and place in an icebox for 24 hours.

While still cold, decant and filter the supernatant fluid into a chemically clean hard-glass bottle.

The yield is about 300 c.c.

Discard the precipitate of potassium perchlorate by washing down the sink with hot water.

Keep the chloric acid in a cool, dark place.

Stock Ceric Sulphate Solution.—

Place 65 gm. of anhydrous ceric sulphate into a 600 c.c. beaker.

Add 60 c.c. of concentrated sulphuric acid.

Mix, and add slowly, while stirring, 50 c.c. of distilled water.

Heat over a low flame at just below the boiling point for 30 minutes.

Let cool.

Add, while stirring, enough distilled water to bring the volume to about 700 c.c.

Filter into a hard-glass or plastic bottle.

This stock solution is sufficient for about a year's supply.

Working Ceric Sulphate Reagent.—

The concentration required is determined by a spectrophotometer reading obtained as follows:

Select a series of 5 test tubes.

In the first, place 1 c.c. of stock ceric sulphate solution.

Add 3 c.c. of 2 N sulphuric acid (60 c.c. of concentrated sulphuric acid per liter in distilled water).

In each of the remaining tubes, place 2 c.c. of 2 N sulphuric acid.

Mix the contents of the first tube and transfer 2 c.c. of the mixture to the second tube.

Mix and transfer 2 c.c. to the third tube.

Continue in this manner until all 5 dilutions have been made. This gives a series of dilutions of 1:4, 1:8, 1:16, 1:32, and 1:64.

In a series of 5 cuvettes, place 1 c.c. of each of the dilutions.

Add to each 5 c.c. of 2 N sulphuric acid.

Mix and read the transmittance in a Coleman spectrophotometer at 420 m μ . Select the dilution that gives a reading between 15 and 20% transmittance.

Should none of the readings come within these limits, take the nearest reading as a guide and make further dilutions until one is found that will give the required reading.

The final dilution will contain ceric sulphate in a concentration of about 0.02 N and will bring the readings in the determinations to the most accurate part of the spectrophotometer scale. Once the proper dilution of the stock solution has been found, it may be employed in all future dilutions.

0.2 N Arsenious Acid.—

Place 9.89 gm. of arsenic trioxide in a 600 c.c. beaker.

Add 70 c.c. of 10% sodium hydroxide.

Stir until dissolved.

Dilute to about 400 c.c. with distilled water.

Add a few drops of phenolphthalein indicator.

Slowly add concentrated sulphuric acid until colorless. Add 45 c.c. of concentrated sulphuric acid in excess, cool, and dilute to 1 liter.

The reagent is completed by addition of sodium chloride as follows:

The method of using sodium chloride, in the ceric sulphate-arsenious acid reaction, differs widely. Zak and his co-workers add NaCl to a ceric ammonium sulphate reagent and use 1.8 mg. in the determination. O'Neal and Simms use 10% NaCl in 10 N sulphuric acid and utilize 50 mg. NaCl in the test. Kirk¹⁰ puts the NaCl in the arsenious acid and adopts still other values. Lein and Schwartz¹¹ have found that the larger amounts of chloride, up to 100 mg. of NaCl, tend to give a more nearly linear relationship with the iodine concentration. In the present procedure, the NaCl is added to the arsenious acid. Since all sodium chloride contains traces of iodine, the amount added to the reagent depends on the quantity of iodine contamination present in the available salt. Before use, all reagents used in the colorimetric system, especially the NaCl, must be tested for this impurity. The procedure recommended by Moran¹² is as follows:

Method of Using Sodium Chloride:

In a series of 3 matched cuvettes, place the reagents as shown in Table 41.

Mix and make transmittancy readings in the spectrophotometer at 420 m μ , immediately, and at 20 and 60 minutes.

Read tubes 1 and 2 at room temperature against tube 3, the water blank. There should be no appreciable change in readings at the end of 1 hour.

In Table 41, a minimal loss of color is shown by a change of only 2% in the transmittance reading in 60 minutes. The results given in the table were obtained with chemicals satisfactory for use in the determinations.

The same reagents, proved by test to be satisfactory as shown in Table 41, are now utilized to determine the suitability of the sodium chloride.

Use a 2% aqueous solution of NaCl of the highest purity available.

Use 4 matched cuvettes to carry out the procedure as shown in Table 42.

Record transmittance readings as before. A difference of not more than 7% between the immediate and 60-minute readings should be satisfactory. A glance at Table 42 shows that 5 mg. is probably the concentration.

Add 250 mg. of NaCl to each 100 c.c. of the arsenious acid solution, which will give the desired concentration of 5 mg. in each 2 c.c. of the arsenious acid reagent to be used in the determination.

Apparatus and Equipment.—

Flat-bottom digestion tubes: These should be about 5 inches by 1 inch, with a graduated mark at the 10 c.c. level, and should be capable of fitting the large cups of the hospital type centrifuge.

A low temperature electric hot plate.

A fume hood.

Technic.—

Place 1 c.c. of serum in the flat-bottom digestion tube.

Add 10 c.c. of 15% trichloroacetic acid.

Mix and centrifuge for 10 minutes at about 2,000 r.p.m.

Pour off and discard the supernatant fluid.

Add 10 c.c. of trichloroacetic acid.

Mix well with a glass rod and thoroughly break up the packed protein precipitate.

Centrifuge for 10 minutes.

Pour off and discard the supernatant fluid.

Allow the inverted tube to drain for a few minutes, and, while waiting, prepare the blank and standards.

Blank and Standards: Select 4 digestion tubes.

Label them 1 to 4.

The first is the blank.

Place 0.5 c.c. of the working standard in the second tube.

Place 1 c.c. of the working standard in the third tube.

¹⁰Kirk, P. L.: *Quantitative Ultramicroanalysis*, New York, 1950, John Wiley & Sons, Inc.

¹¹Lein, A., and Schwartz, N.: *Anal. Chem.* 23: 1507, 1951.

¹²Moran, J. J.: *Anal. Chem.* 24: 378, 1952.

Place 1.5 c.c. of the working standard in the fourth tube.

This gives the standards containing 0.05, 0.10, and 0.15 μg . Since 1 c.c. of serum is used, the standards are equivalent to 5, 10, and 15 μg per 100 c.c. of serum.

In each of the four tubes and the unknown with the serum precipitate put a glass bead.

Pipette 1 c.c. of 0.5% sodium chromate reagent.

Add 10 c.c. of 28% chloric acid.

Stir the precipitate in the serum sample tube with a glass rod and leave the rod in the tube.

Place all the tubes on the low-temperature hot plate, close the hood, and allow to boil for about 1 hour. No attention is needed during this time. A test for the right temperature may be made by placing a piece of white writing paper on the hot plate for about 30 minutes. A slight browning should be all that is observed. If the paper burns, the temperature is too hot, and boiling may be too rapid, with possible loss of iodine.

At the end of 1 hour, observe the tubes, and if the volume has decreased to the point where fumes are beginning to appear, add a drop of chloric acid to each tube.

As the volume approaches 1 or 2 c.c., add a drop of chloric acid at about 5- or 10-minute intervals to prevent loss of iodine. If at any time the yellow color of the chromate turns to green, iodine has been lost and the determination is spoiled.

As the end of digestion is reached (about 2 to 2½ hours) and the volume is 1 c.c. or less, a red precipitate of chromium trioxide will appear. As soon as this is seen, remove the tube from the hot plate and allow to cool.

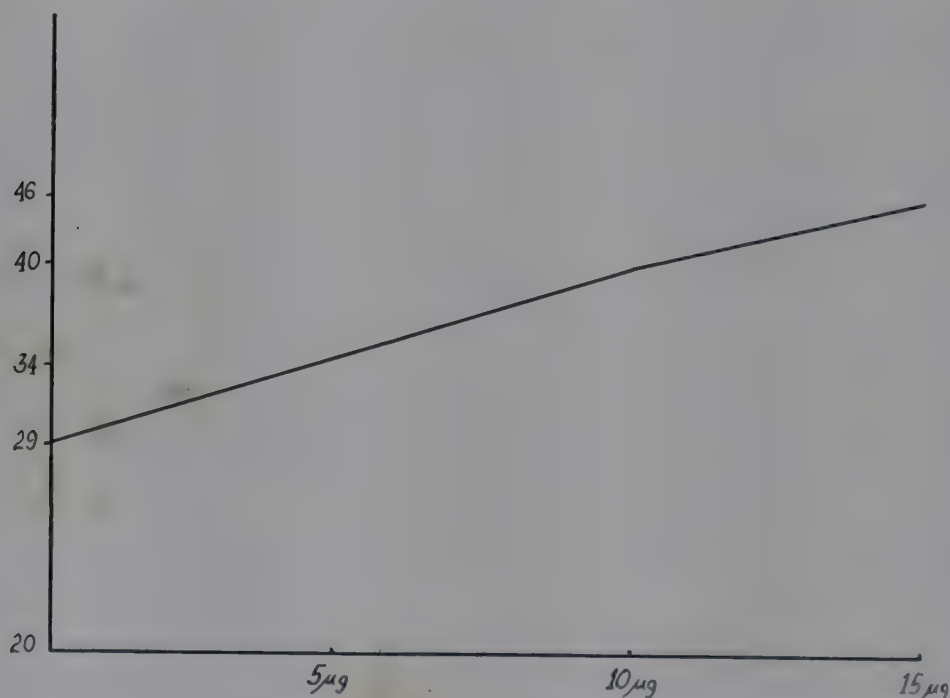


Fig. 134.—Transmittance readings as ordinates plotted against μg per 100 c.c. as abscissas give almost a straightline graph and present a simple method of determining the values for protein-bound iodine.

Add distilled water to the 10 c.c. mark, mix gently, and let stand a few minutes for any precipitate to settle.

Determine the iodine content on 3 c.c. of the digest as follows:

Determination of Iodine Content:

In a series of 5 cuvettes labeled 1 to 5, place 3 c.c. of each of the respective digests.

Add 2 c.c. of arsenious acid reagent to each tube.

Place in the water bath at 30° C. for 20 minutes.

Then, at 30-second intervals, add, from a 1 c.c. rapid delivery pipette graduated to the tip, 1 c.c. of ceric sulphate to each tube.

Immediately replace in the 30° C. water bath.

At the end of exactly 10 minutes from the time the ceric sulphate was added to the first tube, begin reading in the spectrophotometer at 420 $m\mu$ in the same order at 30-second intervals.

Record the transmittance reading at the end of 10 minutes and again at the end of 20 minutes.

The first series of readings is taken to pick out any very high values. The second series, the 20-minute readings, serve to determine the iodine values as follows:

On semilog paper, plot the 20-minute transmittance readings of the standards as ordinates and the equivalent micrograms of iodine concentrations as abscissas. This will give a curve on which the value of the unknown can be found.

TABLE 41.—SIMULTANEOUS TESTING OF 0.2 N ARSENIOUS ACID, 0.02 N CERIC SULPHATE, AND DISTILLED WATER, FOR INTERFERING CONTAMINANTS, SHOW NONE. REAGENTS IN TUBES 1 AND 2 ARE READ IN THE COLEMAN UNIVERSAL SPECTROPHOTOMETER AT 420 Mμ AGAINST BLANK IN TUBE 3

TEST OF REAGENTS FOR INTERFERING CONTAMINANTS*

REAGENTS TESTED	TUBE 1 C.C.	TUBE 2 C.C.	TUBE 3 C.C.
Arsenious acid 0.2 normal	2	1	0
Distilled water	3	4	6
Ceric sulphate 0.02 normal	1	1	0

SPECTROPHOTOMETRIC TRANSMITTANCY READINGS			
Immediate	18	18	100
20 minutes	20	20	100
60 minutes	20	20	100

*Equal loss of color in tubes 1 and 2 shows ceric sulphate contamination. Greater loss of color in tube 1 indicates contamination of arsenious acid. A moderately greater loss of color in tube 2 means water contamination.

TABLE 42.—THE NUMBER OF MILLIGRAMS OF NaCl TO BE ADDED TO EACH 2 C.C. OF SALT-FREE 0.2 N ARSENIOUS ACID IS DETERMINED BY SELECTING THE TUBE THAT GIVES NOT MORE THAN 7 PER CENT DIFFERENCE IN TRANSMITTANCE BETWEEN THE IMMEDIATE AND 60-MINUTE READING. TUBE 1 WITH 10 MG. NaCl GIVES TOO GREAT A DIFFERENCE; SO 5 MG. SHOULD BE TRIED

TEST FOR THE SUITABILITY OF THE SODIUM CHLORIDE*

TUBE NUMBER	1	2	3	4
SODIUM CHLORIDE, MG.	10	20	40	60
REAGENTS	C.C.	C.C.	C.C.	C.C.
Sodium chloride 2%	0.5	1	2	3
Distilled water	2.5	2	1	0
Arsenious acid 0.2 N	2	2	2	2
Ceric sulphate 0.02 N	1	1	1	1

SPECTROPHOTOMETRIC TRANSMITTANCY READINGS AT 420 Mμ				
Immediate	18	18	18	18
20 minutes	21	22	24	25
60 minutes	27	30	35	40

*The 60-minute readings in this test show so much contamination of the sodium chloride that the larger amounts recommended cannot be used.

If the reading of the unknown is greater than that of the 15 μg standard, then a smaller amount of the unknown digest should be taken and colorimetric determination be run again. The 20-minute readings treated in this manner have been compared with the average minute change according to O'Neal and Simms¹² and the results have been found to show close agreement. Only a fraction of a microgram variation has been noted.

A representative protein-bound iodine determination is shown in Table 43, and Fig. 134 illustrates the graph from which the values for the serum samples were obtained.

The sodium chloride tested as shown in Table 42 was used in the determinations given in Table 43. The concentration was 5 mg. in each 2 c.c. of the arsenious acid reagent. The blank gave an ideal reading, and the standard curve is almost a straight line, as seen in Fig. 134. If a lower temperature had been selected for the color reaction, a slightly larger amount of sodium chloride added to the arsenious acid would be necessary to give the same blank reading. As the temperature in the laboratory has been found

¹²O'Neal, L. W., and Simms, E. S.: Am. J. Clin. Path. 23: 493, 1953.

to vary so widely, the 30° C. water bath was selected as being the most convenient. It is very important that a constant temperature be used, and in making the readings in the spectrophotometer, the tubes should be out of the water bath as short a time as possible. Each tube should be out the same length of time and all tubes should be treated exactly alike.

TABLE 43.—A REAGENT BLANK, TUBE 1, AND 3 STANDARDS, TUBES 2, 3, AND 4, ARE ALWAYS RUN WITH THE FOLLOWING TUBES OF SERUM SAMPLES. THE 20-MINUTE READINGS ARE EMPLOYED IN THE CALCULATION OF THE UNKNOWN VALUES
A REPRESENTATIVE PBI DETERMINATION WITH 4 UNKNOWN^{*}

TUBE NUMBER	1	2	3	4	5	6	7	8
TUBE DESIGNATION	B	5 μG	10 μG	15 μG	A34	A35	A36	A37
10-minute reading	23	23	30	31	21	26	25	24
20-minute reading	29	34	40	46	43	37	34	31
Values of unknowns, μg					12.5	7.6	5.0	2.1

^{*}Readings are in per cent transmittance; μg values were obtained from the graph.

It is very important that the patient have none of the following drugs for 2 weeks preceding the determinations: iodine in any form, mercurial diuretics, ACTH and cortisone, bromides, thiouracil, thiocyanates, sulfonamides, desiccated thyroid extract, and thyroxine. All these lead to results that are either too high or too low, depending on the drug used.

The **normal values** by this method seem to be between 4 and 8 μg per 100 c.c. of serum.

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BROMINE IN BLOOD

Yates¹ advises the following technic for determining bromide content in blood:

Apparatus.—

The aeration apparatus is similar to that of Hartner (1933) and consists of a 100 c.c. conical flask fitted with a two-holed rubber stopper. This carries a tube reaching down to the bottom corner of the flask and drawn out slightly at the end. Maximum aeration of the solution in the flask is thus insured, when the latter is inclined at an angle of 60°, while small particles of solution are not carried over into the exit tube. The generation of carbon dioxide in situ by bicarbonate acid mixtures, as suggested by Hartner, has been found unnecessary. The rubber stopper also carries a short glass tube bent at right angles and connected to a small bubbler. The bubbler used by the author consists of a small test tube (10 cm. by 1 cm.), fitted with a rubber bung carrying an exit tube, and an inlet tube drawn out to a V-neck capillary at the bottom. A distance of not more than 2 mm. separates the bottom of this tube and that of the test tube.

Reagents.—

- Solution No. 1. 10% Sodium Tungstate. (Page 225.)
- Solution No. 2. 2/3 N Sulphuric Acid. (Page 225.)
- Solution No. 3. Concentrated Sulphuric Acid (36N).
- Solution No. 4. 20 Gm. of Chromic Oxide and 40 c.c. of Concentrated Sulphuric Acid in 120 c.c. of Distilled Water.

¹Yates, Edmund Denys: *Biochem. J.* 27: Part II, 1763, 1933.

Solution No. 5. 10% Potassium Iodide Solution. (10 gm. KI in 100 c.c.)

Solution No. 6. 0.5% Starch Solution made according to the directions of Nichols (1929).

Technic.—

A. Treatment of Blood.—Measure 5 c.c. of oxalated blood into a 125 c.c. conical beaker; add 35 c.c. of distilled water and allow the mixture to lyse for 30 minutes.

Add 5 c.c. of 10% sodium tungstate solution, mix, and then slowly and with shaking add 5 c.c. of 2/3 N sulphuric acid. Let stand for 10 minutes.

Transfer the bulk of the chocolate-brown mixture to a centrifuge tube and spin at 3000 r.p.m. for 15 minutes.

Pour off the supernatant liquid through a cotton-wool filter into a measuring cylinder. Place 35 c.c. of the protein-free filtrate in a 100 c.c. nickel crucible with 1 c.c. of 25% potassium hydroxide (bromide-free), and evaporate to dryness on a boiling water-bath (1 hour).

Heat the crucible in an electric oven at 500° for 20 minutes.

Dissolve the white residue in 3 c.c. of water and pour into a 100 c.c. conical flask. Wash out the crucible with two additional 2 c.c. portions of distilled water, making the total volume in the flask equal to 7 c.c. (± 0.1 c.c.). Cool to 18° and then proceed as follows:

B. Estimation of Bromine.—Add slowly down the sides of the flask, with shaking and strong cooling under the tap, 2.5 c.c. of concentrated sulphuric acid. At least 10 minutes should be taken for this procedure, and the first two drops, causing evolution of carbon dioxide, must be added carefully in order to prevent losses of hydrogen bromide in the gas evolved.

Now add to the cold solution (18° C.) 4 c.c. of the chromic-sulphuric acid solution. Allow the solution to run all over the sides of the flask, quickly wipe the top of the flask, and then connect up to the aeration apparatus.

This latter should contain 1 c.c. of potassium iodide solution and 4 drops of starch solution.

Aerate for about 2 hours with a steady stream of air (about 30 c.c. per minute).

Renew the absorption tube, and continue aeration for a further 3 hours.

Titrate the iodine liberated in these tubes with N/1000 thiosulphate.

This method is accurate to within 2 γ (gamma) bromine.

Bromine in Blood, Dixon Method

Dixon¹ states that in the investigation of the blood of psychotic patients, who are supposed to have a greatly lowered blood bromine, it was found that none of the methods available for blood-bromine determination was satisfactory. In particular that of Pincussen and Roman was shown to be subject to gross errors. He advocates a method that has an advantage in that oxidation and titration are carried out in the same vessel and all the oxidizing agent is removed. A small amount of chlorate is unavoidably formed, but this is allowed for by using controls which have been subjected to the same procedure from the start. This method makes it possible to determine bromine in 10 c.c. normal blood.

Reagents.—

Water Distilled from alkali in an all-glass apparatus.

10 N Potassium Hydroxide and 10 N Potassium Carbonate purified so as to contain no bromide by von Fellenberg's (1926) method.

Pure Ethyl Alcohol distilled in all-glass apparatus from potassium hydroxide.

¹Dixon, Theodore Frederic: *Biochem. J.* 28: Part I, 48, 1934.

N Potassium Hypochlorite in N/10 Potassium Hydroxide. Made by passing 71 gm. chlorine gas, cooled and dried, into 1.8 liters of potassium hydroxide containing 2.2 gm. mols. or 123.2 gm. potassium hydroxide, finally making up to 2 liters with water. The potassium hydroxide is contained in a flask cooled in ice while the chlorine is passing through to minimize formation of potassium chlorate.

N Potassium Iodide, preferably made fresh, contained in a brown glass bottle.

N Hydrochloric Acid.

0.5% Starch; acid-washed soluble starch freshly made up each time required.

N Hydrogen Peroxide. Dilute 566 c.c. of 3% H_2O_2 to one liter with distilled water in a liter volumetric flask.

N/500 Sodium Thiosulphate Solution.

The proposed method for blood is as follows: 10 c.c. of oxalated blood are measured into a 300 c.c. flask; 0.8 c.c. 10 N potassium hydroxide, 0.8 c.c. 10 N potassium carbonate, and 30 c.c. water are added, and the whole is heated on the water-bath for 4 hours. The mixture is then transferred to a nickel basin of 10 cm. diameter and evaporated to dryness on the water-bath. The basin is dried at 150-160° for 60 minutes and then heated at 420° in the muffle furnace for 2 hours and finally at 500° for 30 minutes. The contents are ground up with water by means of a glass rod, evaporated to dryness on the water-bath, dried at 150-160° for 30 minutes, and then ignited for 30 minutes at 500°. The grinding with water, drying at 160°, and igniting at 500° for 30 minutes are repeated. During ignition in the muffle the contents of the basin swell up considerably, and there is danger of loss unless the surface of the melt is pricked with a finely pointed glass rod, thus allowing tarry vapors to escape. Then 20 c.c. of water are added to the basin, which is heated for a few minutes on the water-bath. The contents are ground up with a glass rod and then filtered through a 9 cm. Whatman's No. 5 filter paper, previously well washed, into a 9 cm. porcelain basin. The residue is repeatedly extracted with hot and finally cold water until there are about 50 c.c. in the porcelain basin which are then evaporated on the water-bath. The porcelain basin is heated on the water-bath to dryness, carefully dried first at 120° for 15 minutes, then at 150-160° for 15 minutes, and finally ignited in the muffle for 10 minutes at 500°. The contents are dissolved in 15 c.c. water and filtered through a 7 cm. Whatman's No. 42 filter paper into another porcelain basin, the filter being washed several times with N/10 potassium carbonate. The solution is evaporated on the water-bath, and the residue is dried at 150-160° and ignited in the muffle for 5 minutes at 500°. It is then completely dissolved in 1.8 c.c. water and alcohol added 5 c.c. at a time with continuous stirring, the mixture being well rubbed with a round-ended glass rod, until 15 c.c. alcohol have been added. The mixture is stirred until the potassium carbonate passes into a pasty condition when the alcoholic layer is decanted through a Jena glass sintered filter No. 3 into a 30 or 40 c.c. flask. The residue is extracted with two more successive lots of 5 c.c. of alcohol. To the potassium carbonate from which the alcohol has been drained is then added 0.6 c.c. water and the above alcoholic extraction is repeated. The contents of the flask are evaporated on the water-bath to dryness, with addition of a couple of pumice granules to facilitate smooth boiling and, if the flask looks very full, under a stream of nitrogen. The last traces of alcohol are removed by heating at 150-160° for 10 minutes. To the residue are added 2 c.c. water, 1 c.c. potassium hypochlorite and 2.5 c.c. saturated sodium chloride, and the mixture is warmed to about 85° on the water-bath and treated with 1 c.c. saturated boric acid. The flask is removed after 20 minutes and cooled; then 1 c.c. N hydrogen peroxide is added, after which the contents are gently boiled for 5 minutes. The flask is cooled and 1.5 c.c. N hydrochloric acid, 5 drops of starch and 1 c.c. N potassium iodide are added; the solution is titrated against N/500 thiosulphate, using a 3 or 5 c.c. microburet.

1 c.c. N/500 = 26.7γ (gamma) bromine.

The oxidation of the bromide in the presence of chloride is very conveniently carried out in the same flask as the titration so that there is very little chance of loss.

The preliminary heating on the water-bath with water and alkali causes partial, if not nearly complete, hydrolysis of the blood-proteins, and as the author has reason to think some of the bromine in the blood is bound to protein, there is less likelihood of loss, and at the same time the use of huge quantities of alkali with consequent corrosion of dishes and basins is avoided. It also permits use of nickel and porcelain basins for the ashing.

The chief aim in the ashing process is to evaporate most of the tarry matter at 400° and then to reduce the ash so that there is very little carbon left by heating at 500°. Grinding up the ash with water and igniting three successive times at 500° seem to remove any possibility of organic matter coming through to the hypochlorite stage. If organic matter is present it will lead to low figures. The method of ashing will not recover substances like bromobenzene or ethyl bromide which are immiscible and evaporate before fixation, but there is no reason to believe that such substances exist in blood.

The contents of the flask after the addition of the potassium hypochlorite must not be boiled as there is danger of excessive potassium chlorate formation. The titration end-point is quite sharp, but, owing to the large salt content of the solution, the color change is from blue-purple to colorless. The blank determination on the 10 c.c. sucrose solution control which is put through exactly the same process is of the order of 0.4 c.c. of N/500 thiosulphate and is due to a small amount of chlorate forming during the heating at 85° on the water-bath and includes the inevitable overtitation so that these both cancel out.

Iodides are also quantitatively oxidized by hypochlorite to iodates and are thus estimated together with bromide.

Normal blood, however, contains 500-1000 γ (gamma)/100 c.c. bromine and 10-15 γ (gamma)/100 c.c. iodine, and thus variations in the latter unless very large, as for instance after iodine therapy, do not appreciably affect the figures for bromine.

LaMotte-Wuth Blood Bromides Comparator*

This is a simple colorimetric test for the rapid and accurate estimation of bromides in the blood, of extreme importance in controlling dosage for prevention of bromide intoxication, delirium, or other symptoms. The test is carried out on deproteinized blood serum, to which gold chloride reagent is added. The color reaction involved is an accurate index of the bromide content, and is measured by comparison against known color standards.

SULFA DRUGS

Determination of Sulfa Drugs in Blood and Urine

With the widespread use of sulfanilamide in various infections, it is necessary to control its administration by a determination of how much is present in blood and urine. Marshall¹ described his present modification as possessing the advantages of being more sensitive, of giving a more stable color, and of requiring fewer manipulations, than the method previously described.² This method consisted of preparing a blood filtrate with toluenesulphonic acid, utilizing the acidity of the precipitant to perform diazotization and coupling with the amine. In determining the conjugated sulfanilamide in blood, the acidity of the blood filtrate is sufficient for hydrolysis on heating.

For the estimation of the sulfanilamide content of the blood of patients receiving the drug, plasma or serum can be used instead of whole blood as there is no essential difference in the values obtained.

*Wuth: J. A. M. A. 88: 2013, 1927.

¹Marshall, E. K., Jr.: Proc. Soc. Exper. Biol. & Med. 36: 422, April, 1937.

²Marshall, E. K., Jr., Emerson, Kendall, Jr., and Cutting, W. C.: J. A. M. A. 108: 953, 1937.

The method described by Bratton and Marshall¹ for the determination of free sulfanilamide in filtrates of blood, urine, and various body fluids can also be used for any derivative of sulfanilamide in which the amino group is free or can be freed by hydrolysis. The reaction depends upon the presence of an amino group substituted in the benzene ring. The method is based on the diazotization of the para-amino-benzene-sulfonamide with nitrous acid, destroying the excess nitrous acid, and then coupling the resulting diazo compound with N-(1-naphthyl) ethylenediamine dihydrochloride.² A purplish-red azo dye is produced which can be readily estimated colorimetrically.

The color reaction is extremely sensitive, so that the drug can be detected in a dilution of 1 part per million or more.

There are two different colorimetric procedures for determining the concentration of sulfa drugs, one of which makes use of the photoelectric colorimeter. This is the more accurate method; the use of the ordinary colorimeter entails a probable error of from 2 to 5 per cent under ordinary conditions, but is applicable to clinical diagnosis.

Methods of Determining Sulfa Drugs in Blood and Urine

The method below gives the details of determining such drugs as sulfanilamide, sulfathiazole, sulfapyridine, or any of the sulfonamide compounds, in blood. It can be used with any of these drugs simply by substituting a standard made of the drug being tested, and following the method given here, or by using the sulfanilamide standard and multiplying the results by the conversion factor given below.

For Blood

Reagents.—(Keep in the refrigerator to avoid decomposition.)

15% Trichloroacetic Acid.—

Dissolve 15 gm. trichloroacetic acid (reagent grade) in distilled water and dilute to 100 c.c. with distilled water.

0.1% Solution of Sodium Nitrite.—

Dissolve 100 mg. sodium nitrite, c. p., in distilled water and dilute to 100 c.c. with distilled water. This solution must be freshly prepared each week.

Aqueous Solution of N-(1-naphthyl) Ethylenediamine Dihydrochloride.²—

Dissolve 100 mg. N-(1-naphthyl) ethylenediamine dihydrochloride in distilled water in a 100 c.c. volumetric flask and dilute to 100 c.c. with distilled water. This solution must be kept in a dark bottle.

0.05% Aqueous Solution of Saponin.—

Dissolve 0.5 gm. saponin in distilled water in a liter volumetric flask and dilute to 1000 c.c. with distilled water.

4 N Hydrochloric Acid.—(See page 24.)

0.5% Aqueous Solution of Ammonium Sulfamate.—

Dissolve 0.5 gm. ammonium sulfamate in distilled water in a 100 c.c. volumetric flask and dilute to 100 c.c. with distilled water.

Stock Solution of Sulfa Drugs.—

Dissolve 200 mg. of c.p. sulfanilamide in 800 c.c. boiling distilled water in a liter volumetric flask. Cool and dilute to 1000 c.c. with distilled water. One c.c. contains 0.2 mg. sulfonamide.

If sulfadiazine is used, dissolve 200 mg. in 500 c.c. distilled water containing 8 c.c. N/1 NaOH before diluting to 1000 c.c.

Stock solutions remain stable for several months if kept in the refrigerator.

¹Bratton, A. C., and Marshall, E. K., Jr.: *J. Biol. Chem.* 128: 537, 1939.

²This chemical may be obtained from the LaMotte Chemical Products Co., Baltimore, Md.

Stock Solution of Sulfadiazine.—

Dissolve 200 mg. of sulfadiazine
 in 500 c.c. of distilled water containing 8 c.c. of N/1 sodium hydroxide
 Dilute to 1000 c.c. with distilled water.

Working Standards of Sulfa Drugs.—

To 10 c.c. portions of 15% trichloroacetic acid in a 100 c.c. volumetric flask add
 5 c.c., 2.5 c.c., and 1 c.c. amounts of the stock solution.

Dilute to 100 c.c. with distilled water.

These standard solutions then contain 1.0, 0.5, and 0.2 mg. per 100 c.c., respectively.

Make these diluted standards just before performing the test.

Technic.—

Pipette 30 c.c. of saponin solution to an Erlenmeyer flask.

Add 2 c.c. of oxalated blood to the saponin, and luke the blood by rotating the flask.

Set aside for 1 or 2 minutes.

Add 8 c.c. of trichloroacetic acid solution.

Filter after precipitation is complete, collecting all of the filtrate.

Determination of Free Sulfonamide.—

To a large test tube labeled "U" for unknown, pipette 10 c.c. of filtrate.

To three similar size test tubes labeled "S₁," "S₂," and "S₃" for the three standards, pipette 10 c.c. portions of the various standard solutions. (Standard I contains 1 mg. per 100 c.c.; Standard II contains 0.5 mg. per 100 c.c.; and Standard III contains 0.2 mg. per 100 c.c.) If testing for sulfanilamide, use the sulfanilamide standard; if testing for sulfaguanidine, use the sulfaguanidine standard, etc.

Add 1 c.c. of 0.1% sodium nitrite to each of the four tubes.

Set aside for 3 minutes. This time is important.

Add 1 c.c. of the ammonium sulfamate solution to each of the four tubes.

Set aside for 2 minutes.

Add 1 c.c. of the ethylenediamine dihydrochloride solution to each of the four tubes.

Compare in a colorimeter. The colors are stable and the comparison may be made immediately or after an hour.

Determination of Total Sulfonamide.—(Free and conjugated.)

Place 10 c.c. of filtrate, prepared as above, in a Pyrex test tube.

Add 0.5 c.c. of 4 N hydrochloric acid.

Place the tube in boiling water bath for 1 hour. Cool and adjust the volume to 10 c.c. Then follow the same technic as outlined above under "Free Sulfonamide."

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{Strength of Standard in mg.} \times \frac{100}{\text{c.c. blood used}} = \text{mg. of sulfonamide in 100 c.c. blood.}$$

Strength of Standard.—S₁ is 0.1; S₂ is 0.05; S₃ is 0.02.

C.c. of blood used-0.5 c.c.

Conversion Factors for Use in Determining Any Sulfa Drug Using a Sulfanilamide Standard

If, instead of using different sulfa drugs to prepare the standards, the sulfanilamide dye is used for all, conversion factors may be used to determine any of the sulfa drugs. These conversion factors are based on the anhydrous molecular weights, which are as follows:

Sulfanilamide	172	(C ₆ H ₅ N ₂ O ₂ S)
Sulfapyridine	249	(C ₁₁ H ₁₁ N ₃ O ₂ S)
Sulfathiazole	255	(C ₉ H ₉ N ₃ O ₂ S ₂)
Sulfaguanidine	214	(C ₇ H ₁₀ N ₄ O ₂ S)

Sulfadiazine.....	250	(C ₁₀ H ₁₀ N ₄ O ₂ S)
Sulfamethylthiazole.....	269	(C ₁₀ H ₁₁ N ₃ O ₂ S ₂)
Sulfasuxidine.....	355	(C ₁₃ H ₁₃ N ₃ O ₂ S ₂)
Sulfapyrazine.....	250.27	(C ₁₀ H ₁₀ N ₄ O ₂ S)
Sulfamerazine.....	264.3	(C ₁₁ H ₁₂ N ₄ O ₂ S)

Example: When sulfaguanidine is being determined by comparison with a sulfanilamide standard, multiply the final results by $\frac{214}{172}$.

For Urine

If urine contains protein, dilute it and treat by the procedure for blood.

If the urine is protein-free, proceed as follows:

Technic.—

Dilute protein-free urine to contain about 1 to 2 mg. of sulfa drug per 100 c.c.

Dilute 50 c.c. of the diluted urine

and 5 c.c. of 4 N hydrochloric acid

to 100 c.c. with distilled water.

Use 10 c.c. of the product of this second dilution as a blood filtrate and test for free sulfa drug as outlined above.

Heat 10 c.c. without further addition of acid and test for total sulfa drugs as above.

Calculations must consider the dilution of the urine.

Determination of Sulfapyridine and Sulfathiazole and Other Sulfa Drugs in Blood and Urine Using the Photoelectric Colorimeter¹

Reagents.—

These are the same as are used in the method given on page 500.

Technic.—

This is a precise method suitable for experimental study or research.²

The standard used for the determination of sulfapyridine is made from sulfapyridine, while that used for the determination of sulfaguanidine is made from sulfaguanidine. Otherwise the methods for any of these drugs are identical.

Use the dilute sulfapyridine, dilute sulfaguanidine, or other sulfa drug standards to establish a calibration curve with the photoelectric colorimeter.

For Blood

Place 1 c.c. of oxalated blood in a flask.

Dilute with 41 c.c. saponin solution.

Allow to stand 1 or 2 minutes.

Precipitate with 8 c.c. trichloroacetic acid solution.

Filter after precipitation is complete.

Determination of Free Sulfapyridine or Free Sulfathiazole.—

In a large test tube, pipette 10 c.c. filtrate.

Add 1 c.c. 0.1% sodium nitrite.

Set aside for 3 minutes. This time is important.

Add 1 c.c. of the ammonium sulfamate solution.

Set aside for 2 minutes.

¹From brochure entitled, "Sulfathiazole" Revised August, 1940, Merck and Company, Rahway, N. J.

²Marshall described a very sensitive and easily performed method for the determination of sulfapyridine which has been applied by Van Dyke et al., to the determination of sulfathiazole (Proc. Soc. Exper. Biol. & Med. 42: 410, 1939).

Marshall, E. K., Jr.: J. Biol. Chem. 128: 537, 1939.

Marshall, E. K., Jr., and Litchfield, J. T., Jr.: Science 88: 85, 1938.

Marshall, E. K., Jr.: J. Biol. Chem. 122: 263, 1937.

Add 1 c.c. of the ethylenediamine dihydrochloride solution.

Read in a photoelectric colorimeter with filter No. 540. The color may be read immediately. The color will not fade for one hour or more.

Determination of Total Sulfapyridine or Total Sulfathiazole.—

Place 10 c.c. of filtrate, prepared as above, in a Pyrex tube.

Add 0.5 c.c. of 4 N hydrochloric acid.

Place the tube in boiling water bath for 1 hour. Cool and adjust the volume to 10 c.c.

Then follow the same technic as outlined above under "Free sulfapyridine and sulfathiazole."

Dilutions of blood of 1:50 or 1:100 may be used. The blood is diluted with water (saponin is unnecessary), allowed to stand a few minutes, and precipitated with trichloroacetic acid solution to the amount of one-fifth of the volume of the blood-water mixture. This permits the use of 0.1 or 0.2 c.c. samples of blood, which are measured with washout pipettes.

When only a very small amount of blood is available, as in the case of small animals such as mice, a determination may be made with considerable accuracy on 0.02 c.c. The adaptation is essentially that described by Marshall and Cutting, the dilution of blood being 1:200 or 1:400, depending on the concentration of sulfathiazole present. The proportion of reagents used is the same as in the other adaptations of the method. Centrifugation before filtration of the protein precipitate is useful in securing the maximum amount of filtrate.

The blood blank is extremely low and negligible for most purposes. With a 1:50 dilution of human blood the correction due to the blood blank varies from 0 to 0.03 mg. per 100 c.c. This blank may be determined easily by performing an analysis as usual, except that water is substituted for the sodium nitrite solution. When small concentrations of sulfathiazole are to be determined or when a dye such as prontosil or neoprontosil is present, this procedure is quite useful. The color of the normal urinary pigments may be conveniently corrected for by the same procedure.

For Urine

If the urine contains protein, dilute it and treat by the procedure for blood.

If it does not contain protein, proceed as follows:

Technic.—

Dilute the urine to contain about 0.2 to 0.5 mg. of sulfapyridine or sulfathiazole per 100 c.c.

Dilute 50 c.c. of this diluted urine
with 5 c.c. of 4 N hydrochloric acid
to 100 c.c. with distilled water.

Treat 10 c.c. of the second dilution as a blood filtrate for free sulfapyridine or free sulfathiazole.

Heat 10 c.c. without further addition of acid and treat as a blood filtrate for total sulfapyridine or total sulfathiazole.

General Facts About This Method.—Determinations on other body fluids are easily made after appropriate dilution.

The reagent blank on distilled water is quite low, but increases with time if the solution is left in the light. For this reason, solutions to be read in the photoelectric colorimeter should be protected from light unless the reading is made immediately.

Some reaction occurs between the trichloroacetic acid and the N-(1-naphthyl) ethylenediamine, since solutions acidified with hydrochloric acid do not show an increased color on exposure to light.

In using a photoelectric colorimeter a filter is essential. With dimethyl- α -naphthylamine-dihydrochloride the peak of the absorption of the azo dye formed occurs at 530 m μ . When N-(1-naphthyl) ethylenediamine-dihydrochloride is used, the peak of absorption is shifted to 545 m μ , and the dyes from sulfathiazole and N-ethanolsulfanilamide show the same absorption peak.

Sulfathiazole in Blood.—

It is indicated in the contribution by Sunderman and Pepper¹ that the estimation of sulfathiazole in whole blood applying the Marshall and Litchfield method indicates that 14 per cent of the drug was lost when an arbitrary amount of it was added to whole blood and then analyzed. This loss occurred during the precipitation of proteins with trichloroacetic acid. Therefore, by the use of serum instead of whole blood, loss of sulfathiazole is considerably less, averaging only 3 per cent. Sunderman and Pepper offer the following method for the analysis in serum in which an arbitrary correction for the loss is made in the final analysis.

Sunderman and Pepper Method for Analysis of Sulfathiazole in Serum Principle.—

Since the recovery of sulfathiazole from serum is within approximately 3 per cent of the theoretical, analyses made on serum make use of an arbitrary correction for the loss encountered. This method is essentially an adaptation of the Marshall and Litchfield procedure for the determination of sulfanilamide in whole blood.

Technic.—

Take blood from the vein in the usual manner, allow to clot, loosen the clot with a wooden applicator, centrifuge, and obtain the clear supernatant serum.

Place 10 c.c. of 8% trichloroacetic acid in a 25 c.c. volumetric flask.

Add 2 c.c. blood serum.*

Shake thoroughly to keep the precipitate finely divided and dilute to 25 c.c. with distilled water.

Mix thoroughly and allow to stand for 20 minutes.

Filter through Whatman No. 44 filter paper to obtain a clear, colorless filtrate.

Diazotize 10 c.c. of the filtrate at room temperature with

1 c.c. of 0.1% sodium nitrite (fresh).

Allow to stand for 3 minutes.

Add 1 c.c. of 0.5% ammonium sulfamate.†

Mix thoroughly.

Add 5 c.c. dimethyl- α -naphthylamine‡ coupling reagent.

Stopper. Invert once. Allow to stand for 10 minutes.

For colorimetric standards use sulfathiazole standards of appropriate concentrations carried through the same procedure as the unknown (0.2, 0.4, and

*In blood serum containing high concentrations of sulfathiazole, 1 c.c. of serum is employed.

†Marshall and Litchfield recommend that the solution of ammonium sulfamate be buffered with sodium dihydrogen phosphate in order to increase the rate of coupling. In our experience the addition of the buffer has not proved necessary.

‡Solution contains 1 c.c. dimethyl- α -naphthylamine in 250 c.c. of 95 per cent ethyl alcohol.

¹Sunderman and Pepper: Am. J. M. Sc., Dec., 1940.

0.8 mg. per 100 c.c., respectively, prepared from stock solution of sulfathiazole containing 200 mg. per liter).

Shake the solutions well before placing them in the colorimeter cups so as to eliminate small gas bubbles which may be present. (The gas is presumably nitrogen formed by destruction of HNO_2 with $\text{NH}_2\text{SO}_3\text{NH}_4$.)

A colorimeter with an attachable lamp may be used for comparisons.

Fit the eyepiece of the colorimeter with a No. 74 Wratten filter.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{Mg. of Sulfathiazole in Standard} \times \text{Dilution Factor} \times \text{Correction}$$

Factor = mg. per 100 c.c.

Correction factor = 1.03.

Practically quantitative recovery of sulfathiazole from protein-free urine can be made with this method, disregarding the correction factor. Make appropriate dilutions of the urine so that the sulfathiazole concentration in the diluted urine is between 5 and 30 mg. per 100 c.c.

So far as analysis of urine is concerned, a number of writers have called attention to the great danger in the use of the drug by the occurrence of crystalline structure in the urinary passages. Accordingly, they studied the excretion of the drug in urine, its solubility, and crystalline structure. They found that both sulfathiazole and its acetyl derivative were about twice as soluble in urine of pH 7.6 as in urine of pH 5.6; hence it may be inferred that when crystalline concentrations owing to sulfathiazole therapy threaten, an effort should be made to keep the urine alkaline and to secure a large urinary volume.

Determination of Sulfapyridine, Ratish and Bullova. (Bedside Test)

Ratish and Bullova¹ described what they term a bedside test for sulfapyridine. The concentration of sulfapyridine in the blood is determined by the rate of absorption, the rate of excretion, and the rate of conversion. Absorption, excretion, and conversion vary independently and are influenced by unknown factors. Unless the parts per thousand in the blood or milligrams per hundred cubic centimeters are known, clinical failure cannot be ascribed either to inefficiency or to inadequate concentration of the drug. When the concentration is too low, larger and more frequent oral doses or parenteral administration may be employed.

Their test is a modification of the Marshall test and can be carried out in ten minutes.

The following reagents and apparatus are required for the determination of sulfapyridine by this method.

Reagents.—

Ether.

15% Trichloroacetic Acid Solution.—See page 500.

0.1% Solution of Sodium Nitrite.—See page 500.

1% Solution of Urea.—

A solution of α -dimethyl-naphthylamine containing 1 c.c. in 250 c.c. of 95% ethyl alcohol. (This reagent should be kept in a dark dropping bottle.)

¹J. Lab. & Clin. Med. 25: 645, 1940.

Apparatus.—

2 c.c. Luer syringe and needle.

1 test tube of 20 c.c. capacity with round bottom, stoppered with cork and marked at 1 c.c. and 6 c.c.

1 centrifuge tube with long taper, graduated to 15 c.c. in 0.1 c.c. divisions, fitted with rubber stopper.

5 dropping bottles to contain the reagents listed above.

A comparator block 2.5 inches by 4.5 inches, with 4 openings to take test tubes 100 by 12 mm., and color standards.*

Technic.—

Using a Luer syringe, withdraw approximately 1.5 c.c. of venous blood.

Invert the syringe with the needle still attached and deliver the blood, drop by drop, into the round-bottomed test tube to the 1 c.c. mark.

Add ether to the 6 c.c. mark, using the other dropping bottle.

Insert the stopper and shake vigorously for 2 minutes. The fluids will separate rapidly into two layers, with the ethereal extract of sulfapyridine in the upper layer.

Slowly release the stopper.

Cautiously decant the ethereal extract into the centrifuge tube to the 0.5 c.c. mark and set the extraction tube aside for duplicate tests.

By means of a dropping bottle, add 15% trichloroacetic acid solution to the 5 c.c. mark.

Place the rubber stopper over the mouth of the tube and shake vigorously for 10 to 20 seconds.

Add 0.5 c.c. of 0.1% sodium nitrite solution (7 to 8 drops from a dropping bottle), bringing the solution to the 5.5 c.c. mark.

Again shake vigorously for 20 seconds.

Add 0.5 c.c. of 1% urea solution dropwise from a dropping bottle.

Add α -dimethyl-naphthylamine to the 8.5 c.c. mark from its dropping bottle, close the tube with the rubber stopper, and invert once or twice. The white opalescence is soon replaced by a purplish red color.

The color is fully developed after five minutes, and the tube may be compared with standards in the comparator block at that time.

All values of this ether extraction method lie within 75 to 95 per cent of those obtained by the Marshall method, using a photocolormeter.

The quantitative estimation of sulfapyridine depends upon the formation of a purplish red azo dye. This diazotization may be completed in approximately one minute and the full color developed in five minutes.

The rate of color formation after coupling with α -dimethyl-naphthylamine, the comparative action of ammonium sulfamate and urea on the destruction of the excess nitrite, and the value of trichloroacetic acid and paratoluenesulfonic acid as media for the reaction were studied. With a photoelectric colorimeter results were obtained which indicated that either group of reagents may be used. Urea and trichloroacetic acid are recommended because they are cheaper and more readily obtainable.

(Note.—While ether does not extract sulfapyridine from watery solutions or urine as efficiently as it does from blood, it extracts sulfapyridine well from cerebrospinal fluid.)

Preparation of Color Standards

Color standards may be readily prepared using phenol red. They are calibrated so that the value of sulfapyridine obtained from the sample corresponds to the true value. Dissolve 0.0075 gm. of phenol red in 100 c.c. of distilled water. To a series of 5 tubes, add 3.9 c.c. of fifteenth molar potassium dihydrogen phosphate (KH_2PO_4) and 6.1 c.c. fifteenth molar secondary sodium phosphate (Na_2HPO_4). Then follow with 0.2 c.c. of normal sodium hydroxide (NaOH) and phenol red as indicated. The volume of the phenol red should increase progressively in the different test tubes.

*Reading is facilitated when the width of the color band is increased by placing tubes filled with water behind the standard and the test.

The phenol red solutions are transferred to test tubes 100 by 12 mm. and sealed. The value of each tube should be marked on or permanently attached to it. The standards should be protected from direct sunlight. New standards should be prepared every six months.

AMOUNT OF PHENOL RED	AMOUNT OF SULFAPYRIDINE (TRUE VALUE) INDICATED
0.22 c.c.	Concentrations up to 4 mg. per cent.
0.30 c.c.	Concentrations up to 7 mg. per cent (usual in blood of recovered pneumococcic pneumonia patients).
0.46 c.c.	Concentrations up to 10 mg. per cent.
0.62 c.c.	Concentrations up to 12.5 mg. per cent (desirable in blood of meningitis patients).
0.86 c.c.	Concentrations up to 15 mg. per cent.

Eimer and Amend, 205-223 Third Avenue, New York, N. Y., furnish the outfit needed to carry out this test.

LaMotte Sulfonamides Outfit¹

This utilizes a method of determining sulfanilamide, sulfapyridine, sulfathiazole, sulfaguanidine, sulfadiazine, and all of the other sulfa compounds that react to produce a color in the diazo coupling reaction.

The principle is based upon the chemical formation of a brilliant dyestuff, when the reagents are added to a specimen of blood or urine, the intensity of this developed color being representative of the concentration of the sulfa compound. Two-tenths cubic centimeter of blood from the finger or ear, or the same amount of urine, is required. Venous blood may also be used. The actual technic involves laking the blood with saponin solution, removing the proteins, and diazotizing the filtrate with sodium nitrite. Ammonium sulfamate is used to remove the excess nitrite, and when the coupling agent N(1-naphthyl)ethylenediamine is added a brilliant red color results, which is compared with color standards of known concentrations of the sulfa compound.

Determination of p-Aminobenzoic Acid, Conjugated p-Aminobenzoic Acid, and p-Nitrobenzoic Acid in Blood

There is a close structural relationship between sulfanilamide and p-amino-benzoic acid. The Marshall method is commonly used for the determination of sulfanilamide. This method consists in diazotizing a trichloroacetic acid blood filtrate for 2 or 3 minutes with 0.1 per cent sodium nitrite, destroying excess nitrous acid with 0.5 per cent ammonium sulfamate solution containing potassium acid phosphate (KH_2PO_4) as a buffer, and finally developing a red color suitable for colorimetric determination by coupling the diazotized solution with dimethyl- α -naphthylamine. Eckert² described a modification of this method which he found very satisfactory for the determination of p-amino-benzoic acid. He changed the Marshall method by: (1) increasing the concentration of sodium nitrite from 0.1 to 0.2 per cent; (2) by increasing the concentration of ammonium sulfamate from 0.5 to 2.0 per cent; (3) by using no buffer at all; and (4) by lengthening the diazotization period.

Apparatus.—

Klett-Summerson photoelectric colorimeter with green filter No. 56 and matched test tubes of 12.5 mm. diameter.

15 by 150 mm., lipped Pyrex test tubes graduated at 10 c.c. and 12 c.c.

¹Marshall, E. K., Jr., and Bratton, A. C.: *J. Biol. Chem.* 128: 537, 1939.

²Eckert, H. W.: *J. Biol. Chem.* 148: 197-203, 1943.

Reagents.—**Sodium Nitrite, 0.2 Per Cent.—**

Dissolve 200 mg. of sodium nitrite, c. p., in a volumetric flask, in enough distilled water to make 100 c.c. solution. This solution must be freshly prepared.

Approximately 4N Hydrochloric Acid.—

Add 320 c.c. of concentrated hydrochloric, c. p., to distilled water in a liter volumetric flask, and dilute to 1000 c.c. with distilled water.

Ammonium Sulfamate (LaMotte), 2 Per Cent.—

Dissolve 2 grams of ammonium sulfamate (LaMotte) in distilled water in a 100 c.c. volumetric flask and dilute to 100 c.c. with distilled water. This is much stronger solution than is necessary, but assures complete destruction of excess nitrite during the analysis.

Dimethyl- α -Naphthylamine (Eastman).—

Add 1 c.c. of the Eastman reagent to 95 per cent alcohol, and dilute to 250 c.c. with 95 per cent alcohol.

Titanous Chloride (LaMotte), 20 Per Cent, Standardized.—**Trichloroacetic Acid.—**

15 per cent solution.—Dissolve 15 grams of trichloroacetic acid (Merck) in enough distilled water to make 100 c.c. solution.

2.7 per cent solution.—Dilute 18 c.c. of the 15 per cent solution to 100 c.c. with distilled water.

***p*-Aminobenzoic Acid Solution.—**

Dissolve 100 mg. of the *p*-aminobenzoic acid in 100 c.c. of 2.7 per cent trichloroacetic acid solution. Use Eastman chemicals after recrystallizing from water. Dilutions for standards or recovery experiments in blood are made from these stock solutions.

***p*-Nitrobenzoic Acid Solution.—**

Dissolve 100 mg. of *p*-nitrobenzoic acid in 100 c.c. of 2.7 per cent trichloroacetic acid solution. Use Eastman chemicals after recrystallizing from water. Dilutions for standards or recovery experiments in blood are made from this stock solution.

Sulfanilamide Solution.—

Dissolve 100 mg. sulfanilamide (Winthrop) in 2.7 per cent trichloroacetic acid in a 100 c.c. volumetric flask and dilute to 100 c.c. with 2.7 per cent trichloroacetic acid.

Tartaric-Hydrochloric Acid Mixture.—

Add 36 gm. of tartaric acid (Eastman) and 42 c.c. of concentrated hydrochloric acid to 100 c.c. of distilled water. This solution has a slight yellow color which does not interfere with the test.

Technic.—**(A) Preparation of Blood Filtrates.—**

Measure 2 c.c. of oxalated or citrated blood into a 50 c.c. flask.

Add 30 c.c. of distilled water, and mix thoroughly. Allow laking to take place for 5 minutes.

Add 8 c.c. of 15 per cent trichloroacetic acid slowly with rotation. Shake the mixture vigorously and allow to stand 15 minutes.

Filter through filter paper. The filtrate is used for the determination of *p*-aminobenzoic acid, *p*-acetylaminobenzoic acid, and *p*-nitrobenzoic acid.

(B) Determination of *p*-Aminobenzoic Acid in Blood Filtrates.—

A blank must always be run along with the test. The blank for this determination, and also that for the conjugated (acetylated) *p*-aminobenzoic acid, consists of 10 c.c. of 2.7 per cent trichloroacetic acid solution treated exactly as the filtrate.

Filtrate.—Place 10 c.c. of the blood filtrate in a 50 c.c. flask.

Add 2 c.c. of distilled water.

Add 1 c.c. of 0.1 per cent sodium nitrite solution and allow the mixture to stand for from 15 to 20 minutes.

Add 1 c.c. of 2 per cent ammonium sulfamate, mix well, and let stand for from 2 to 3 minutes.

Finally, add 5 c.c. of the alcoholic dimethyl- α -naphthylamine and allow to stand for from 30 to 60 minutes in order to develop the maximum color. Read in the colorimeter with the blank set at zero.

(C) Determination of Conjugated (Acetylated) *p*-Aminobenzoic Acid.—

Place 10 c.c. of the blood filtrate in a graduated Pyrex test tube and add 0.5 c.c. of 4 N hydrochloric acid.

Place the tube in a boiling water bath for 1 hour, cool to room temperature, and dilute the mixture with distilled water to the 10 c.c. mark.

Transfer to a 50 c.c. flask and rinse the tube with 2 c.c. of distilled water, adding this rinsing to the first solution.

Now treat the solution exactly as in the case of the free *p*-aminobenzoic acid.

(D) Determination of *p*-Nitrobenzoic Acid in Blood Filtrates.—

Place 10 c.c. of the blood filtrate in a graduated Pyrex test tube and add 1 c.c. of tartaric-hydrochloric acid mixture.

Add 2 drops of 20 per cent titanous chloride.

The function of the tartaric-hydrochloric acid mixture is to prevent the formation of insoluble titanous acid on heating.

Mix well by rotating and then place in a boiling water bath for 10 to 15 minutes.

Cool the tube rapidly to room temperature and dilute with distilled water to the 12 c.c. mark.

Transfer to a 50 c.c. flask.

Add 1 c.c. of 0.2 per cent sodium nitrite solution. Mix, and let stand for 20 minutes with occasional swirling. This last step removes the remaining traces of titanous ion and also diazotizes the amine.

Add 1 c.c. of 2 per cent ammonium sulfamate. Mix, and allow to stand for 2 to 3 minutes.

Finally, add 5 c.c. of alcoholic dimethyl- α -naphthylamine and allow the color to develop for 4 to 5 hours before reading. This longer time is necessary for maximum color development, since the rate of coupling is greatly reduced in the presence of the additional reagents. The mixture may stand overnight at this point, without danger of fading. If the colored solution is not clear, centrifuge.

Blank.—Prepare a blank by using 10 c.c. of 2.7 per cent trichloroacetic acid solution treated exactly as the sample.

Read in the colorimeter against the blank set at zero.

Standards.—

A standard containing 0.025 mg. of *p*-aminobenzoic acid or an equivalent amount of sulfanilamide may be used. It is treated exactly as described under the determination of *p*-aminobenzoic acid. Sulfanilamide is a convenient standard, since in solution it keeps indefinitely in a cool dark place.

Calculations.—

With the Klett-Summerson photoelectric colorimeter, readings are directly proportional to the concentration of the substance being determined: the factor equals the amount of standard divided by the scale reading. To calculate free *p*-aminobenzoic acid, multiply the scale reading by the factor, with suitable corrections for the relative molecular weights if sulfanilamide was used as the standard. (Multiply by 0.796.) Conjugated *p*-aminobenzoic acid is determined by comparing the amounts found before and after hydrolysis.

In determination of *p*-nitrobenzoic acid, subtract the amounts of free and conjugated *p*-aminobenzoic acid from the total amount found after reduction with titanous chloride. Convert the difference, representing *p*-aminobenzoic acid derived from *p*-nitrobenzoic acid, to the equivalent weight of *p*-nitrobenzoic acid by dividing by 0.82, then divide the value so found by 0.788 to correct for the fact that recovery was only 78.8 per cent of the theory in horse blood filtrates.

(E) Determination of *p*-Aminobenzoic Acid in Culture Media.—

For the determination of *p*-aminobenzoic acid in broth cultures, pass the cultures through a porcelain filter.

Mix 2 c.c. samples of the filtrate with 8 c.c. of distilled water and 2 c.c. of 15 per cent trichloroacetic acid.

Carry out the determination as described under blood filtrates.

Centrifuge the sample until clear. It is then ready to be read in the colorimeter with a suitable blank set at zero.

The determination of *p*-nitrobenzoic acid in broths or other media presents special difficulties. If the material is cloudy and cannot be easily cleared by centrifuging, as in the case of pneumococcus cultures, treat as previously described and allow the color to reach a maximum. Then add some Celite¹ or similar substance to clarify the suspension. Centrifuge the colored solution until clear; cap the centrifuge tubes to prevent evaporation. The colored solutions should not be filtered, since as much as 20 to 25 per cent of the color may remain on the paper. In the presence of the concentrations of phosphate ordinarily in culture media (0.1 to 0.2 per cent), the method for the determination of *p*-nitrobenzoic acid is not applicable; but the limiting concentration of phosphate has not been determined.

When colored broths are analyzed, a blank on the untreated broth must be determined.

Specificity and Sensitivity.—Certain chemicals, such as phenol, cresol, *m*- and *o*-aminobenzoic acids, aniline, sulfanilamide compounds, and many other related substances give color reactions by this method but are generally not found in biologic fluids. It might be mentioned here that peptone broths sometimes give a slight color under the conditions of the method and this interference has been traced to the presence of tryptophane.² Blood filtrates from normal animals give no reaction.

The reaction described is extremely sensitive and 1 γ of *p*-aminobenzoic acid can easily be determined in 10 c.c. of filtrate. By suitable reduction of volumes a still greater sensitivity may be attained and it is possible to determine 0.1 γ in 1 c.c. of filtrate if special small colorimeter tubes are used.

ESTIMATION OF BLOOD THIOCYANATES³**Method of Barker**

Thiocyanates are used in the treatment of hypertension. The dosage must be controlled chemically because thiocyanates produce toxic manifestations, such as, fatigue, weakness, mental confusion, disorientation, anemia, and dermatoses. Best results are obtained when there is a concentration of from 8 to 12 mg. per cent; toxic manifestations may be encountered when the thiocyanate concentration exceeds 15 to 30 mg. per cent. Barker suggests that blood thiocyanate determinations should be made because toxic symptoms appear when blood concentrations reach 35 to 60 mg. per 100 c.c.

Reagents.—**10% Trichloroacetic Acid Solution.—**

Dissolve 10 gm. trichloroacetic acid, c.p., in enough distilled water to make 100 c.c.

Ferric Nitrate Reagent.—

Dissolve 50 gm. ferric nitrate, crystallized, in
500 c.c. distilled water.

Add 25 c.c. concentrated nitric acid

Dilute to 1000 c.c. with distilled water.

¹Celite analytical filter aid (Johns-Manville).

²Eckert, H. W.: J. Biol. Chem. **148**: 205 (1943).

³Barker, M. H.: J. A. M. A. **106**: 762, 1936.

Stock Thiocyanate Solution.—

Dissolve 1 gm. of potassium thiocyanate in
800 c.c. distilled water.

Titrate a 20 c.c. portion of a standard silver nitrate solution (2.9195 gm. silver nitrate in 1 liter of distilled water) acidified with 5 c.c. of concentrated nitric acid, with the potassium thiocyanate solution, using ferric ammonium sulphate as an indicator. Calculate the amount of water necessary to add to the potassium thiocyanate solution to make 20 c.c. equivalent to 20 c.c. of silver nitrate solution. Add the calculated amount of distilled water, mix thoroughly, and check the solution by further titrations. (See pages 26 and 27 for exact details.)

Standard Solutions of Thiocyanate.—

Standard I. (5 c.c. solution contains 0.5 mg. thiocyanate ion.)

Dilute 100 c.c. of the stock thiocyanate solution to 1000 c.c. with distilled water in a liter volumetric flask.

Standard II. (5 c.c. solution contains 0.35 mg. thiocyanate ion.)

Dilute 70 c.c. of stock thiocyanate solution to 1000 c.c. with distilled water in a liter volumetric flask.

Standard III. (5 c.c. solution contains 0.2 mg. thiocyanate ion.)

Dilute 40 c.c. stock thiocyanate solution to 1000 c.c. with distilled water in a liter volumetric flask.

Technic.—**Unknown**

Place 5 c.c. of 10% trichloroacetic acid solution in a test tube.

Add 5 c.c. serum or plasma.

Stopper and shake well.

Allow to stand from 10 to 15 minutes; then filter through a small filter paper.

The filtrate should be perfectly clear. If it is not, filter again through the same filter paper.

Place 5 c.c. of the filtrate in a clean dry test tube labeled "U" for unknown.

Add 1 c.c. of ferric nitrate reagent.

Standard

Place 5 c.c. of each of the three standard solutions in its respective test tube properly labeled S_1 , S_2 , or S_3 .

Add 5 c.c. trichloroacetic acid solution to each.

Add 2 c.c. ferric nitrate reagent to each.

Mix the three standards and the unknown well and read in a colorimeter with the standard set at 20 mm., choosing the standard which most nearly matches the color of the unknown.

Calculation.—

If S_1 was used, and standard set at 20;

$$\frac{200}{\text{Reading of Unknown}} = \text{mg. thiocyanates in 100 c.c. serum.}$$

If S_2 was used, and the standard set at 20;

$$\frac{140}{\text{Reading of Unknown}} = \text{mg. thiocyanates in 100 c.c. serum.}$$

If S_3 was used, and standard set at 20;

$$\frac{80}{\text{Reading of Unknown}} = \text{mg. thiocyanates in 100 c.c. serum.}$$

LaMotte Thiocyanate Outfit

Determination of thiocyanates in blood is useful for controlling the dosage of thiocyanate when used in the treatment of hypertension. The procedure is based on the fact that a deep brownish color is obtained when a specimen of deproteinized blood serum or plasma

containing thiocyanates is treated with a ferric nitrate solution. The reading is made by visual comparison against permanent color standards, representing concentrations of thiocyanate from 4 to 20 mg. per 100 c.c. of blood.

DETERMINATION OF ASCORBIC ACID IN BLOOD

Principle.—

Farmer and Abt¹

The proteins in the blood plasma are precipitated by metaphosphoric acid. The supernatant liquid is titrated with dichlor-phenol-indo-phenol sodium.

Apparatus.—

- 5 c.c. buret graduated in 0.02 c.c.
- 2-15 c.c. centrifuge tubes.
- 4-2 c.c. pipettes.

Reagents.—

2,6-Dichlor-Phenol-Indo-Phenol Sodium Solution.*—

Dissolve 1 tablet (corresponding to 1 mg. of vitamin C) of 2,6-dichlor-phenol-indo-phenol sodium in distilled water in a 100 c.c. volumetric flask and dilute to 100 c.c. with distilled water. Each c.c. of this solution corresponds to 0.01 mg. of vitamin C. This solution may be used for three days if kept in a refrigerator.

5% Metaphosphoric Acid Solution.—

Dissolve 5 gm. metaphosphoric acid in distilled water in a 100 c.c. volumetric flask and dilute to 100 c.c. with distilled water. Keep in a refrigerator.

Technic.—

Place 2 c.c. of plasma obtained from oxalated blood in a 15 c.c. conical centrifuge tube. Add 4 c.c. distilled water and mix.

Add 4 c.c. of 5% metaphosphoric acid solution and mix.

Centrifuge and remove the supernatant fluid.

Transfer 2 c.c. of the supernatant fluid to a centrifuge tube.

Titrate with 2,6-dichlor-phenol-indo-phenol sodium solution from a buret into the fluid in the centrifuge. The first permanent faint pink color is the end point. Read from the buret the number of c.c. of 2,6-dichlor-phenol-indo-phenol solution used.

Calculation.—

C.c. dichlor-phenol-indo-phenol sodium used $\times 0.01 \times \frac{100}{0.4}$ = mg. of ascorbic acid per 100 c.c. of blood plasma.

Or, multiply the reading on the buret by 2.5 to obtain the mg. of ascorbic acid per 100 c.c. plasma.

Normal.—0.8 to 2.4 mg. per 100 c.c. plasma. Renal threshold is approximately 1.4 mg. per 100 c.c. plasma. Clinical scurvy 0.1 to 0.5 mg. per 100 c.c. of plasma.

Determination of Reduced Ascorbic Acid in Small Amounts of Blood For Detection of Vitamin C Intake

Method of Farmer and Abt²

Farmer and Abt have developed a micromethod for determination of quantity of reduced ascorbic acid in blood plasma. This depends upon the dietary supply of vitamin C, or of ascorbic acid administered as a medicament.

*The tablets may be obtained from Hoffmann-LaRoche, Inc., Nutley, N. J., or from many jobbers. E. H. Sargent and Co., Chicago, can supply the dye tablets. For standardization of the dye when research is in progress, titration against Cebione, Merck (crystalline vitamin C), is recommended. This is placed on the market in 0.1 gm. vacuum-sealed ampules.

¹Farmer, C. J., and Abt, A. F.: Proc. Soc. Exper. Biol. & Med. 32: 1625, 1935; 34: 146, 1936.

²Farmer, C. J., and Abt, A. F.: Proc. Soc. Exper. Biol. & Med. 32: 1625, 1935.

Abt, A. F., Farmer, C. J., and Epstein, I. M.: J. Pediat. 8: 1, 1936.

Farmer, C. J., and Abt, A. F.: Proc. Soc. Exper. Biol. & Med. 34: 146, 1936.

Equipment.—

Microburet
 Pipette
 Titration tile
 Blood collection phial
 Tube

Reagents.—**Neutral Potassium Oxalate****5% Metaphosphoric Acid Solution (freshly made)**

Dissolve 5 gm. of metaphosphoric acid in enough distilled water to make 100 c.c.

Standard Dye Solution for Clinical Work

(Sodium 2,6-dichlor-phenol-indo-phenol)

Place 1 tablet of sodium 2,6-dichlor-phenol-indo-phenol² in a 50 c.c. volumetric flask.

Dissolve in distilled water, then dilute to 50 c.c. with distilled water. Mix thoroughly.

1 c.c. of this dye solution is equivalent to 0.02 mg. ascorbic acid.

Standardized Solution of 2,6-dichlor-phenol-indo-phenol.—Each c.c. of the solution is equivalent to approximately 0.02 mg. of ascorbic acid, but the solution should be standardized against pure ascorbic acid or other suitable standard before use each day. The solution is stable for about one week if stored in a dark bottle in the refrigerator. The presence of a small amount of phosphate buffer, pH 6.8, increases the stability of the dye solution. A standard thiosulphate solution provides a good means of standardizing the dye.¹

2.5% Metaphosphoric Acid (freshly made)

Dissolve 2.5 gm. metaphosphoric acid in enough distilled water to make 100 c.c.

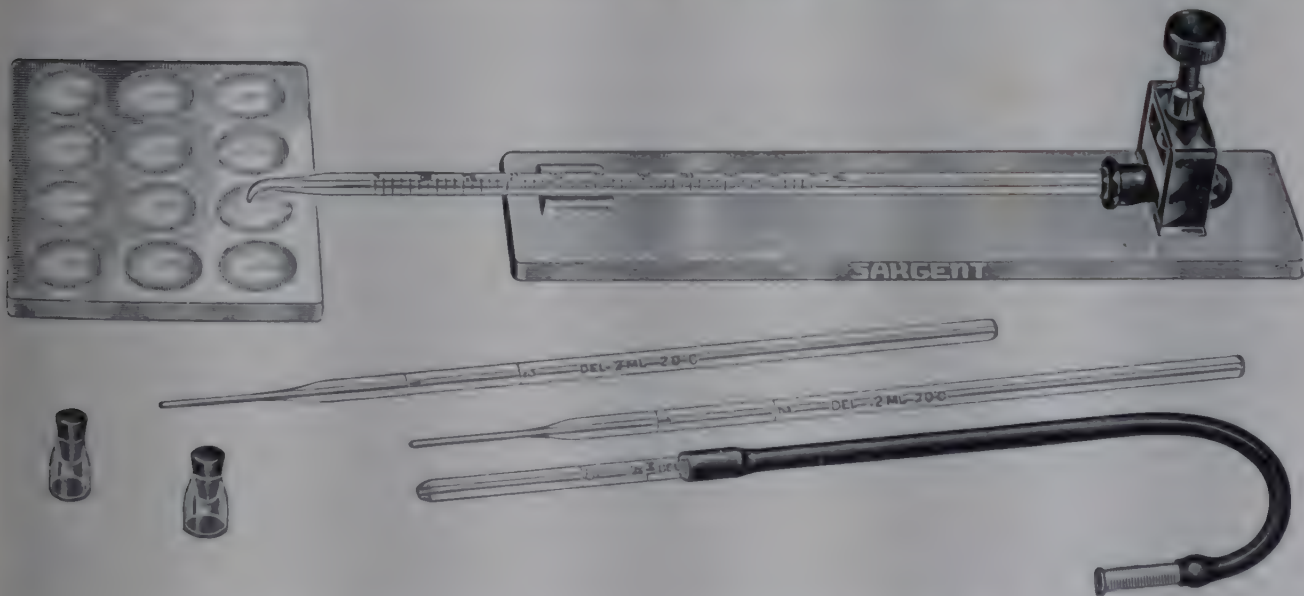


Fig. 125.—Apparatus for Farmer-Abt method of determining reduced ascorbic acid. (Courtesy E. H. Sargent & Co., Chicago, Ill.)

Technic.—

Obtaining the Blood. Prick the finger, and collect 6 to 8 drops (0.3 to 0.4 c.c.) blood in the phial, which contains a small amount of potassium oxalate. Stir with a toothpick, stopper, and centrifuge.

Deproteinization.—Pipette 0.1 c.c. plasma plus 0.1 c.c. water plus 0.2 c.c. fresh 5% metaphosphoric acid solution into a 15 c.c. conical centrifuge tube. Mix thoroughly. Centrifuge down the coagulated protein.

Titration.—Titrate with sodium 2,6-dichlor-phenol-indo-phenol. Fill the microburet from dye solution placed in a clean test tube held nearly horizontally when slipped over

¹Menaker, M. J., and Guerrant, N. B.: *Indust. Engr. Chem., Anal. Ed.* 10: 25, 1938.

the curved end of the buret. Turn the screw to the right until a small drop of mercury is expelled into the dye solution; then fill to the desired point by turning the screw in the reverse direction.

Place 0.2 c.c. of 2.5% metaphosphoric acid in a depression of the titration tile.

Into an adjacent tile depression, pipette 0.2 c.c. of the sample of deproteinized plasma, the ascorbic acid content of which is to be estimated.

Read the microburet. Now titrate the deproteinized plasma until the first discernible trace of color (faint pink) is obtained, in comparison with the metaphosphoric acid solution in the adjacent depression. After taking the reading from the buret, the metaphosphoric acid solution is titrated until the color matches that of the plasma (which has just been titrated).

The buret reading is again taken, and this value is subtracted from the reading of the plasma. Make plasma titrations in duplicate.

Calculation.—

$(\text{c.c. dye} - \text{c.c. blank}) \times S \times 2000 = \text{mg. cevitic acid (reduced form) per 100 c.c. blood plasma.}$

Example.—

Dye used in plasma titration = 0.026 c.c.

Dye used in blank = 0.002 c.c.

$S = 0.02$

$(0.026 - 0.002) \times 0.02 \times 2000 = 0.96 \text{ mg. per 100 c.c. plasma.}$

Interpretation.—

Comparison of reduced cevitic acid content of the blood with the vitamin C intake indicates that they parallel each other.

A reduced cevitic acid content of blood plasma less than 0.50 mg. per cent indicates a marked insufficiency of Vitamin C intake.

The Determination of Ascorbic Acid (Cevitic Acid) (Vitamin C) in Urine

Macromethod. Method No. I*

Collection of Sample: Titrate single specimens immediately. Preserve twenty-four-hour collections by adding enough 12 per cent metaphosphoric acid to the receptacle to maintain a final concentration of 2 to 3 per cent. A less expensive but less certain method is to add 10 c.c. of glacial acetic acid for each 100 c.c. of urine, kept in the refrigerator. 8-Hydroxyquinoline is also an excellent protective agent. Holmes¹ has added carbonate to acidified samples with good results.

Titration.—In titrating urines that are not excessively colored it is preferable to titrate directly into aliquot samples large enough (generally 10 to 100 c.c.) to consume enough of the dye to give good readings. It is less convenient, but sometimes preferable, to titrate the urine from a buret into 5 c.c. of standardized dye solution and 5 c.c. of distilled water in an evaporating dish. When fresh samples are being titrated, acid should be added to give a pH of about 3.0 to 3.5, to minimize interference from reducing substances other than ascorbic acid.²

Determination of Ascorbic Acid in Urine—Method of Farmer and Abt. Method No. II

Urine is suitably diluted and titrated with an aqueous solution of the sodium salt of 2,6-dichlor-phenol-endo-phenol.

Collect a 24-hour sample of urine in a bottle containing 10 gm. of metaphosphoric acid. Refrigerate and as soon as possible undertake the determination.

Measure the total volume and then dilute 5 c.c. with 2.5% metaphosphoric acid (HPO_4) (p. 513) solution until only a faint color remains. This facilitates reading the end point.

*The author is indebted to Merck and Co., Inc., through the courtesy of its Medical Director, Dr. John Carlisle, for permission to publish this procedure.

¹Holmes, H. N., and Campbell, K.: J. Lab. & Clin. Med. 24: 1293, 1939.

²Kastlin, G. J., King, C. G., Schlesinger, G. R., and Mitchell, J. W.: Am. J. Clin. Path. 10: 882-893, 1940.

If the urine is known to contain a high content of ascorbic acid, the dilution should be 1:10 or 1:20. Transfer 2 c.c. aliquots of the diluted urine to test tubes and add 2 c.c. of a 2.5% HPO_4 solution.

Titrate, using a standardized dye solution which is added from a 5 c.c. microburet, and then run a blank determination on the reagents.

Calculation.—

The urinary output of ascorbic acid is calculated as follows: c.c. dye required by 2 c.c. dilute urine - c.c. blank \times dye value $\times \frac{\text{dilution}}{2} \times$ c.c. 24 hr. urine = mg. eliminated in 24 hours.

Urine Determination of Ascorbic Acid—Modified Tillman Method.[†] Method No. III

Place recently standardized dye (2,6-dichlor-phenol-indo-phenol, above) in the 5 c.c. microburet. Place 1 c.c. of urine in a centrifuge tube and titrate rapidly to the first pink color that persists for ten seconds. The end point is best found by matching with a control tube containing urine but no dye. If the urine has not previously been acidified, add 1 to 2 drops of 10% acetic acid to the 1 c.c. portion before titration. In cases in which a large test dose has been given and an increased concentration of cevitamic acid has resulted, the urine must be diluted with distilled water before titration; i.e., after an intravenous test dose of 1 gm. the 1½-hour urine specimen will often require dilutions of 1:50 to 1:100, and the 5-hour specimen may require dilutions to 1:50. For the most accurate titrations this dilution should be adjusted so that less than 2 c.c. of the dye is required to titrate the 1 c.c. portion of urine.

Calculation.—

Mg. cevitamic acid per c.c. dye \times volume of specimen in c.c. \times dilution factor \times c.c. dye used to titrate the 1 c.c. of urine.

Example.—

$0.025 \times 95 \times 50 \times 1.5 = 178.1$ mg. of cevitamic acid in specimen.

Determination of Vitamin C in Urine—Method of Harris and Ray.¹ Method No. IV

Reagents.—

2,6-Dichlor-Phenol-Indo-Phenol Sodium Solution.—

This is a 1 mg. per cent solution. See page 513.

Glacial Acetic Acid.—

Technic.—

Pipette 10 c.c. of the dichlor-phenol-indo-phenol sodium solution into a small casserole. Add 1 to 2 drops of acetic acid until a red color appears.

Titrate from a buret filled with urine until the color of the dye disappears. This is the end point.

Read from the buret the c.c. of urine used.

Calculation.—

$\frac{0.1 \times 100}{\text{c.c. urine used}} = \text{mg. of vitamin C per 100 c.c. urine.}$

Note: It is very important to carry out the titration immediately following the collection of the specimen.

Normal.—25 mg. vitamin C in 24 hours.

LaMotte Outfit for the Determination of Vitamin C in Blood and Urine. Method No. V

This is a colorimetric method for the rapid determination of vitamin C in blood and urine, employing the oxidation-reduction indicator 2,6-dichlor-phenol-indo-phenol.

The method is based on the principle that an excess of the dye 2,6-dichlor-phenol-indo-phenol is added to the deproteinized blood filtrate or urine, and the residual color measured

[†]Tillman, J., Hirsch, P., and Jockisch, J.: *Ztschr. f. Untersuch. d. Lebermitt.* 63: 241, 1932.

¹Harris and Ray: *Lancet* 228: 71, 1935.

by comparing with color standards. The extent of this decolorization of the dye is a measure of the vitamin C content of the blood. Other substances in the blood react with 2,6-dichlorophenol-indo-phenol, but at a much slower rate than vitamin C. Therefore, it is believed that the speed with which the amount of vitamin C is determined in this test offers an advantage over slower titration methods.

CHAPTER IV

HEMATOLOGY

This chapter has been revised with the assistance of
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INTRODUCTION TO HEMATOLOGY

Hematology is one of the most important departments of laboratory work. Through hematologic examinations one is enabled to make diagnoses of many obscure conditions and to follow the course of these conditions. For many years we have been concerned with various hematologic procedures, first, with an examination of unstained blood, later with the use of specific tinctorial methods for the demonstration of various kinds of white cells. Then methods of absolute blood counting were introduced, together with special tests, such as blood sedimentation, cell volume, bleeding time, clotting time, blood typing, oxydase reaction, supravital staining, and a method of differential counting introduced by Victor Schilling.

While technical advances have been made in blood examinations and many new clinical observations have been recorded regarding the various blood dyscrasias, much of our information in the past has come from anatomical investigations.

Recently, however, more important advances have been made since the physiologists and experimental physiologists have together applied themselves to the unknown problems of blood development and its various aberrations. It is strange that with all the work that has been done on the anatomy of the blood and of blood-forming organs and the application of technical methods to the diagnosis of these conditions, the etiology of so many blood diseases remains still unsolved. Within the past fifteen years, immunohematology has become a branch in itself. The investigators in this field have added much to our knowledge of blood dyscrasias. The use of anticoagulants in the treatment of thrombo-embolic disease has stimulated a renewal of interest in the mechanism of blood coagulation. Pauling's work in hemoglobin has opened an entirely new field in molecular disease of hemoglobin. These are but a few examples of the dynamic approach to problems in the field of hematology.

In connection with the contents of this chapter, one must bear in mind that hematology is not a single science but rather a part of clinical medicine. A good hematologist must first and foremost be a skilled clinician capable of using all the diagnostic methods known to medicine necessary to arrive at the correct hematologic diagnosis. This point has been emphasized by Riser¹ in an article

¹Riser, W. H.: *South. M. J.* 40: 153, 1947.

entitled, "The Practical Handling of Hematology by the Clinical Pathologist." He decided very properly that the clinical pathologist, in order to be a good hematologist, must not only know the individual blood cells but he must be skilled in the interpretation and correlation of the clinical laboratory values in the light of the clinical history and physical findings. Not all clinical pathologists are capable hematologists. The clinical pathologist who thinks only in terms of the microscope, test tubes, or pipettes, and completely loses sight of the clinical history and physical findings, cannot be expected to view a blood film through the narrow eyepiece of the microscope and see the complete clinical picture. The general practitioner or internist may interpret the results obtained by a laboratory technician. If he happens to understand the significance of the blood findings, he can be quite useful in making the correct diagnosis. A clinical pathologist, in other words, to be most efficient, must be a special consultant in hematology. He must become a consultant in clinical medicine and must constantly broaden his general knowledge of clinical medicine so as to become really an experienced clinician. Riser stated that the clinical pathologist is often regarded as a highly specialized magician imbued with the power of visualizing the complete blood picture upon study of a single blood spread, without previously having any knowledge of the clinical history or physical findings of the patient. This is clearly a ridiculous situation. The diagnosis can be arrived at only after indicated laboratory studies are completed and careful integration and correlation of all available information have been carried out. Sometimes it is necessary to study the patient over a period of time before the correct diagnosis is evident. The practice of sending blood films to the clinical pathologist with request for hematologic diagnosis is to be decried. It is often necessary for us to write for all the pertinent data before venturing on any conclusions. It is erroneous to use bone marrow studies alone and quite separate from all the other facts of the case as a means of diagnosis. Another excellent hematologist, the late Roy R. Kracke, showed that the subject of hematology is but a division of internal medicine and that a true clinical pathologist must be an eminently accomplished clinician. Kracke stated that for some time he had been very reluctant to give an opinion on a blood film, realizing that the chances of error are considerable when one does not have available all pertinent data concerning the clinical problem. Emphasis is, therefore, to be laid on the fact that, first, a complete study of the blood must be made from a laboratory standpoint and, second, these data must be correlated very carefully by either the clinician or the clinical hematologist in order to arrive at a probable diagnosis. Without minimizing in any way the importance of the following pages devoted to hematology from a laboratory standpoint, one must bear in mind that a technician cannot possibly be a hematologist and that a clinical pathologist cannot be a hematologist unless he is an accomplished clinician.

THE ORIGIN OF BLOOD CELLS

There still is considerable discussion as to the origin and development of the various blood cells. We have studied the embryonic blood cells of man

and the lower animals and have traced their development through various ages. Certain points are still in controversy as to the monophyletic or polyphyletic development of the principal leukocytes.

In 1935, we wrote in the first edition of this book, "There has been considerable dispute as to the origin of the blood cells. The matter is by no means definitely settled." In succeeding revisions of this work, we continued to call attention to the lack of unanimity of opinion on this subject. As a matter of fact, there is very little of the sanguine crusading spirit in recent publications concerning the origin of blood cells that existed in the early literature of hematology. For an extensive review of this subject and bibliography, we recommend that the reader refer to Downey's *Handbook of Hematology*.¹ In this handbook, the various theories of the origin of blood cells have been exceedingly well portrayed in the section by William Bloom.

It is generally agreed that the most primitive blood formation in the early embryo begins with the layer known as "mesoderm." The first cells to be formed in this early period of blood formation are red cells often spoken of as "primitive erythroblasts." In this very early stage, or mesoblastic period, it is very difficult to find cells that correspond to the white cells of the adult. It is also important to remember that at this stage blood-forming organs as we know them in the adult or later stage of the fetus are not present, and much of the blood formation takes place within "blood islands" in the yolk sac of the embryo.

At about the second month of fetal development, there begin to appear cells that correspond to the white blood cells in the adult and, in addition, megakaryocytes can be demonstrated in the liver. At this stage, the liver becomes a blood-forming organ and presents red blood cell formation as well as white blood cell formation. Associated with this activity of the liver is a modest activity of the spleen, which at first is most concerned with erythropoiesis but later is concerned with the formation of lymphocytes. At about the fifth month of fetal development, the final phase of hematopoiesis is instituted in the fetus. At this point the bone marrow begins to inaugurate its ultimate growth as one of the greatest blood-forming tissues in the human body. Simultaneous with this development of the bone marrow, the lymphatic system and lymph nodes begin the development of lymphocytes.

Despite the fact that many studies have been made in the fetus concerning blood development, it is very difficult to transfer the information obtained from observations of the embryo and fetus to the adult human in so far as blood development is concerned. One interesting observation is that in the fetus, the early red cell development or "primitive" development shows cells somewhat similar to the abnormal red cells seen in pernicious anemia. An interesting point about this similarity is that this primitive erythropoiesis gives way to "definitive" erythropoiesis (the adult type of normal erythropoiesis) at the same time that the fetal liver begins to develop. It is rather interesting that with the administration of liver therapy to patients with pernicious anemia ("primitive erythropoiesis"), the erythropoiesis re-

¹Downey, H.: *Handbook of Hematology*, New York, 1938, Paul H. Hoeber, Inc.

verts to "definitive type." Excepting pernicious anemia and anemias of a pernicious-like character, it is difficult to demonstrate in the adult any red cell development that corresponds to the development in the very early embryo.

Regarding the development of leukocytes, there is still considerable disagreement concerning each type of white blood cell, and, as a result, there have appeared several schools of theory concerning the development of leukocytes. Fig. 136 is a chart representation of the various theories of blood formation. These theories, briefly, divide themselves into two major groups, the *monophyletic* and the *polyphyletic* schools.

The monophyletic theory insists that all blood cells, regardless of their character, are derived from a single primitive blood cell that perhaps corresponds to the primitive mesenchyme of the embryo. In the normal adult human being it is very difficult, first of all, to find cells that are identical with primitive mesenchymal cells, and it is even more difficult to identify step-like development of such primitive cells through the various forms of leukocytes and erythrocytes. Another strong position that the monophyletists take is that in the adult there are cells that are totipotent, that is, they have the power of developing in any direction. Although this is the complete monophyletic theory, there are other men (Downey, Pappenheim, and Ferrata) who believe in the monophyletic theory but who do not agree that certain cells are totipotent. These men have been labeled "neo-unitarians" because they feel that once certain cells have become functionally differentiated, they cannot revert to other cell types.

The polyphyleticists can also be divided into several subgroups, such as "dualists" (two series of development of white blood cells), "trialists" (three main stems of development), etc. Ehrlich and Naegeli represent proponents of the dualistic school, and Schilling and others, the trialistic.

These divergent theories are the result of examining different types of material by different avenues of approach. For instance, it is said that Maximow refused to look at any type of pathologic hemopoietic tissue. Others, such as Bloom, who succeeded Maximow, depended to a great extent upon tissue culture for arriving at their viewpoint. Still others were involved in the examination of abnormal blood and blood-forming organs from pathologic human cases. Others tried to carry correspondence between embryonic tissue and adult human tissue for development of their theories, while still others resorted to special stains, such as peroxidase and supravital stains. Animal experiments of varied character also have added to the store of knowledge of hematopoiesis and have played their role in the development of these theories. The important thing to remember is that although there is great divergence of opinion, it is still possible to examine blood and blood-forming organs and generally arrive at a reasonable diagnosis even though one may differ as to terminology. Even in this matter of nomenclature, there is great difference of opinion as to what certain cells should be called, for perhaps several authors are speaking of the same cell by a different name. The subject of nomenclature is discussed below.

Suffice it to say at this point that the almost religious fervor in the crusades for various theories of blood formation is now much more a matter of the historic past than of interest to hematologists.

The various theories as to the origin of blood cells have been exceedingly well portrayed in a monograph by William Bloom.¹ We are indebted to the author of this monograph and to the publisher for permission to quote from this splendid review of this subject and to quote it freely. Bloom states that the so-called "trialism" theory of Schilling is about as follows: There proceeds from the mesenchyme cells myeloblastic, lymphoblastic, and histiocytic cells, the last of which become monocytes.

Of Cunningham, Sabin, and Doan's theory, Bloom states: "They derive lymphocytes, granular leukocytes, and monocytes from fixed reticular cells by way of a small round precursor which they call a primitive blood cell. The formation of granulocytes takes place in the bone marrow, that of monocytes in the spleen, and of lymphocytes in spleen and lymph nodes. These authors also describe the development of the red blood cells and macrophages (clasmatoocytes) from endothelium."

The dualistic theories of Ehrlich, Schridde, and Naegeli derive the blood cells from two sources, myelocytic and lymphocytic.

The extreme unitarians, according to Bloom, are Dominici, A. Wolff, Ciaccio, Maximow, Dantschakoff, Weidenreich, Latta, Jordan, Jolly, Jordan and Speidel, Bloom, Kingsbury, and Maximow and Bloom. They believe that the three-stem cells of the connective, lymphatic, and myeloid tissues of the adult, and of the various sites of blood formation in the embryo are representatives of a single cell type. Maximow adopted the term "hemocytoblast" for the free stem cell.

There are so-called neo-unitarian theories, of which Pappenheim, Ferrata, and Downey are the leading exponents. They uphold the unitarian theory of blood cell formation and admit to a greater or less degree the morphologic identity of the free stem cells in the myeloid and lymphatic tissues. They believe that the cells in their respective situations are functionally differentiated.

It may be well to summarize in tabulated form the various and differing conceptions of the origin of the white blood cells. Wiseman² (Fig. 136) summarizes the principal points that are in conflict and in agreement between the various theories that have been advanced to explain the histogenesis of the blood cells. In another chart (Fig. 137) Wiseman shows the monophyletic interpretation of the literature that is concerned with the relationships between the cells of the blood and those of the tissues.

A recapitulation of the preceding shows blood cells, according to the unitarian group, coming from undifferentiated mesenchymal cells; according to the dualists, the sources are myelocytic and lymphocytic, and some origin from the histiocytes of the tissues. The trialism school, which is headed by Schilling and his followers, believes in three points of development of white cells. Plate I, frontispiece, illustrates the Schilling trialism theory.

¹Bloom, William: Chapter on Lymphocytes and Monocytes: Theories of Hematopoiesis. Handbook of Hematology, Vol. I, Hal Downey, Editor, pages 419-435. Publishers, Paul B. Hoeber, Inc., Medical Book Department of Harper and Brothers, New York, 1938.

²Wiseman, B. K.: J. A. M. A. 103: 1524-1529, 1934.

Maturation	Reticulo-Endothelial System				
Undifferentiated	Reticulo-endothelial cell			Reticulum cell "mesenchymal rest"	Endothelial cell
Partial differentiation	(1 Blast cell) Lymphocyte	(2 Blast cells) Myeloblast Lymphoblast	(3 Blast cells) Myeloblast Lymphoblast Megaloblast	(3 Blast cells) Myeloblast Lymphoblast Monoblast	(1 Blast cell) Megaloblast
Complete differentiation	All blood cells	RBC Granulocyte Monocyte Clasmatocyte Lymphocyte Monocyte Clasmatocyte	RBC Granulocyte Monocyte Clasmatocyte Lymphocyte Monocyte Clasmatocyte	Granulocyte Lymphocyte Monocyte	RBC Clasmatocyte
Hypothesis	Unitarian	Dualist	Trinitarian	Modified dualist with separate macrophage origin.	
School	Monophyletic	Polyphyletic (restricted)			Polyphyletic (Complete)

Fig. 136.—This chart summarizes the principal points in conflict and in agreement between the various theories advanced to explain the histogenesis of the blood cells. There are three levels of maturation common to all; in the first level, primitive undifferentiated mesenchymal cells provide the blast cells of the second level, which in turn, by maturation and division, became the completely differentiated cells of the circulating blood, represented by the third level. The degree of polygenesis is determined by the number of partially differentiated (blast) cells believed to be necessary in order to render accordant the experimental facts and observations as interpreted by the various investigators who comprise the schools of thought shown in the lower portion of the chart. The principal difference, as indicated, is in the degree of prepotency ascribed to the blast cells, the unitarians feeling that a single common ancestor (the lymphocyte) may differentiate into any of the mature types of cells. Attributing lesser degrees of potentialities to this cell results in increasing degrees of polyphyletism, which in the extreme construction requires a separate blast cell for each of the circulating elements, and a separation of the reticuloendothelial system of cells into its component units, each with different potentialities.

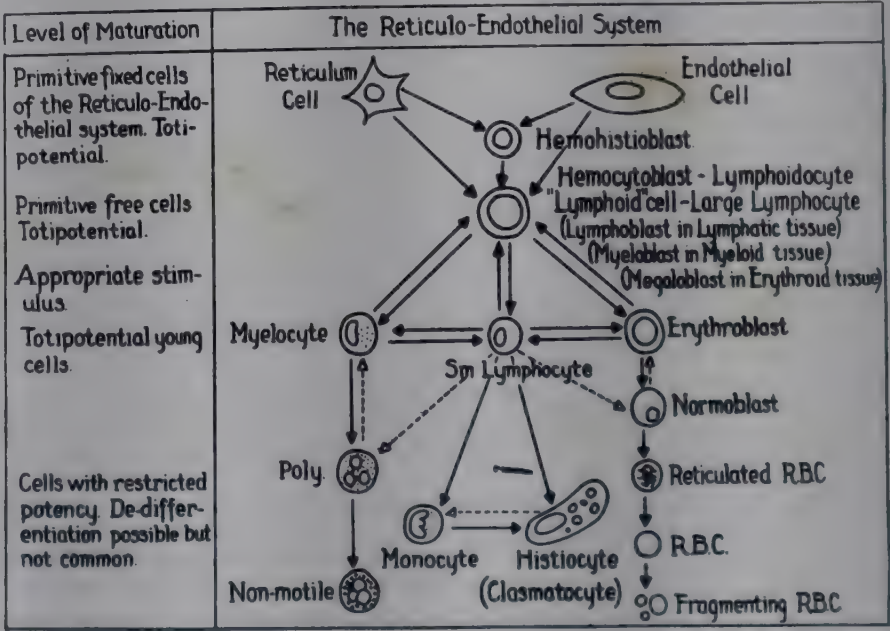


Fig. 137.—This chart condenses, by diagram, the monophyletic interpretation of the literature that is concerned with the relationships between the cells of the blood and those of the tissues. A common undifferentiated totipotential cell is postulated and identified with the hemocytoblast of Ferrata, the lymphoidocyte of Pappenheim, and the blood lymphocyte, lymphoblast, myeloblast, megaloblast, and monoblast of the polyphyletic adherents. In the theory the small lymphocyte of the blood is believed simply to be a temporary resting phase of the larger lymphoid cell and may differentiate into the other cells, as shown, either directly or after reverting back to the large tissue cell of the Ferrata type. The small or large lymphocyte may also develop into the more mature blood cells directly without going through the maturation phases of these cells, and, finally, after having so differentiated, it may dedifferentiate back to the primitive totipotential level. The reticulum and endothelial cells of the reticuloendothelial system are believed to be potentially identical, differing only in shape. They give rise to the lymphocytes of the blood, either directly or through a primitive tissue cell similar to the hemohistioblast of Ferrata. Opinion varies between different supporters of the monophyletic school as to the importance of the transition from monocyte and clasmatocyte back to lymphocyte, as well as other minor points intentionally omitted on this chart, in order to emphasize the important concepts involved in this theory of blood formation.

NOMENCLATURE OF HEMATOLOGY

There is considerable confusion in hematologic literature due to terminology. Osgood suggested a nomenclature for the erythrocyte and granulocyte series because of the disagreement in definitions and the inappropriateness of the terms in current use. He called attention, for instance, to the term *normoblast*, which is a combination of a Latin and Greek root which should mean a normal stem cell since the termination blast is ordinarily employed only for the most immature cells of a series. The cell, however, is neither a stem cell nor a normal cell of the blood. He further maintains very properly that the derivation for the erythrocyte series is from the Greek word meaning *nucleated*. The old term *myeloid series* meaning marrow-like cells is a misnomer because they are cells forming an integral part of the marrow. This does not differentiate them from cells of the monocyte, plasmacyte, or erythrocyte series which are also found normally in the marrow. Osgood believes, as we do, that the term *granulocyte series* is more logical for all cells of this group.

TABLE 44 A.—RECOMMENDED TERMS AND TERMS TO BE AVOIDED WHEN REFERRING TO SPECIFIC CELLS OF THE ERYTHROCYTIC SERIES

TERM TO BE USED	TERMS TO BE AVOIDED
Rubriblast	Erythroblast, megaloblast, pronormoblast, promegaloblast, normoblast, hemocytoblast, stem cell, myeloblast, lymphoidocyte, karyoblast
Prorubricyte	Erythroblast, megaloblast, pronormoblast, normoblast, macronormoblast, macroblast, prokaryocyte
Rubricyte	Normoblast, pronormoblast, macronormoblast, erythroblast, polychromatophilic normoblast, karyocyte
Metarubricyte	Normoblast, erythroblast, metakaryocyte
Reticulocyte*	
Erythrocyte	Red blood cell, erythroplastid, normocyte, akaryocyte

*It is recommended that the reticulocyte stage be considered a subdivision of the erythrocyte stage.

Reproduced from the Am. J. Clin. Path. 19: 56-59, 1949, by courtesy of the Editor and of the Williams and Wilkins Company, Baltimore.

TABLE 44 B.—RECOMMENDED TERMS AND TERMS TO BE AVOIDED WHEN REFERRING TO CELLS OF A PARTICULAR SERIES OR TO A DISEASE AFFECTING ANY CELL OF THAT SERIES

TERM TO BE USED	TERMS TO BE AVOIDED
Lymphocytic	Lymphoid, lymphatic, lymphogenous, lymphocyte, mononuclear
Granulocytic	Myeloid, myelogenous, myelocyte, myeloctytic, granulocyte, leukocyte, leukocytic, leucocyte, leucoctytic
Monocytic	Monocytoid, monocytogenous, mononuclear, monocyte
Plasmacytic	Plasma cellular, plasmacytogenous, myeloma cell, plasmacyte
Erythrocytic	Erythroid, erythrocytoid, erythron, erythrocytogenous, erythrocyte
Thrombocytic	Megakaryocytic, platelet, thrombocyte

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Because of the confusing terminology that has developed in the field of hematology, a special committee of outstanding hematologists from various parts of the United States met in Chicago in October, 1947, for the purpose of attempting to clarify the nomenclature of cells and diseases of the blood and blood-forming organs. The major recommendations are listed in Tables 44 A and B and 45. "It is not the intention of the Committee to imply from

its recommendation of terms to be used that the origin of all these cells has been settled.”

It should be mentioned here that not all of the members of the Committee were in entire accord as to the final nomenclature to be used, but they felt that some modicum of agreement would help eliminate some of the confusion in the field of hematology. Despite the efforts of this group to unify the nomenclature, there are certain terms of historic use that will be difficult to eliminate from the writing and speaking of hematologists the world over.

TABLE 45.—RECOMMENDED TERMS AND TERMS TO BE AVOIDED WHEN REFERRING TO SPECIFIC CELLS OF THE BLOOD AND BLOOD-FORMING ORGANS

NAME OF SERIES	TERM TO BE USED	TERMS TO BE AVOIDED
Lymphocytic	Lymphoblast	Myeloblast, hemocyto blast, lymphoidocyte, stem cell, lymphocyte
	Prolymphocyte	Large lymphocyte, pathologic large lymphocyte, atypical leukocytoid lymphocyte, monocyte, immature lymphocyte
	Lymphocyte	Small, medium or large lymphocyte, normal lymphocyte, small, medium or large mononuclear
Monocytic	Monoblast	Myeloblast, hemocyto blast, lymphoidocyte, lymphocyte, stem cell, immature monocyte
	Promonocyte	Premonocyte, hemohistioblast, immature monocyte, Ferrata cell
	Monocyte	Large mononuclear, transitional, clasmatocyte, endothelial leukocyte, histiocyte, resting wandering cell
Granulocytic	Myeloblast	Granuloblast, hemocyto blast, lymphoidocyte, lymphocyte, stem cell
	Progranulocyte	Promyelocyte II, leukoblast, myeloblast, premyelocyte, promyelocyte, progranulocyte A
	Myelocyte	Granulocyte, myelocyte B, nonfilament, class I
	Metamyelocyte	Metagranulocyte, juvenile, myelocyte C, nonfilament, class I
	Band cell	Staff cell, stab cell, nonfilament, class I, rod nuclear, polymorphonuclear, stabkernige, rhabdocyte, nonsegmented
	Segmented	Polymorphonuclear, filamented, class II, III, IV, or V, lobocyte
Plasmacytic	Plasmablast	Myeloblast, hemocyto blast, lymphoidocyte, lymphocyte, stem cell, lymphoblastic plasma cell, myeloma cell
	Proplasmacyte	Türk cell, Türk irritation form, lymphoblastic or myeloblastic plasma cell, myeloma cell
	Plasmacyte	Plasma cell, Unna's plasma cell, Marschalko's plasma cell, plasmacytoid lymphocyte, myeloma cell
Thrombocytic	Megakaryoblast	Megalokaryoblast
	Promegakaryocyte	Premegalokaryocyte
	Megakaryocyte	Megalokaryocyte
	Thrombocyte	Platelet, thromboplastid
—	Disintegrated cell	Senile cell, smudge, basket cell, smear cell, degenerated cell

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Glossary of Common Hematologic Terms

Hematopoietic organs are the blood-forming organs.
Erythropoiesis is the manufacture of red blood cells.
Erythrocytes are red blood corpuseles.

Normocytosis is a condition characterized by the presence of normal-sized erythrocytes (**normocytes**).

Microcytosis is a condition characterized by the presence of small erythrocytes (**microcytes**).

Macrocytosis is a condition characterized by the presence of large erythrocytes (**macrocytes**).

Anisocytosis is abnormal variation in the size of erythrocytes.

Poikilocytosis is the appearance of abnormally shaped erythrocytes: pear-shaped, oak-leaf, club, crenated.

Crenation is the scalloped or notched appearance of the edges of erythrocytes found in pathologic conditions, or caused by undue pressure when making a blood film. It is also noted when erythrocytes are suspended in hypertonic solutions.

Normochromia or **normochromasia** refers to normal pigment content of erythrocytes.

Hypochromia or **hypochromasia** refers to decreased pigment content of erythrocytes.

Hyperchromia or **hyperchromasia** refers to increased pigment content of erythrocytes.

Polychromasia is the presence in the fixed blood film of blue-gray-staining erythrocytes.

Basophilic punctation or **basophilic stippling** is a characteristic of mature erythrocytes which, under certain toxic conditions, show dark staining of punctate bodies in the cytoplasm, demonstrable by routine staining methods.

Reticulocyte is a slightly immature red cell containing a dark-staining web or lace-like structure, *which can be demonstrated only by some type of supravital staining*.

Basophilia of the erythrocytes is the appearance in the circulating blood of immature erythrocytes which show bluish gray staining of the cytoplasm demonstrable by routine staining technics.

Normoblast is a nucleated red blood cell.

Megaloblast is an abnormal "primitive" nucleated red blood cell.

Hemacytometers are instruments used in counting blood cells.

Anemia is a symptom-complex accompanied by reduction in red blood cells, hemoglobin, and red cell volume per cent of variable degree.

Oligocytosis, or **hypoglobulia**, or **erythropenia**, or **oligocythemia** is a low red blood cell count.

Erythrocytosis, or **polycythemia**, or **hyperglobulia** is an elevated red blood cell count.

Rouleaux formation of the erythrocytes is the tendency of the erythrocytes to lie with overlapping edges, or in the "roll" formation such as may be seen when a stack of coins is pushed over.

Hemoglobin is an iron-bearing protein which gives the red color to blood.

Hemoglobinometers are instruments used for measuring hemoglobin.

Oxyhemoglobin is the bright red hemoglobin that is loosely combined with oxygen, and is found in the arteries.

Reduced hemoglobin is a combination of hemoglobin and carbon dioxide, and is found in venous blood.

Leukopoiesis is the manufacture of white blood cells.

Leukocytes are white blood cells.

Leukocytosis is a high white blood cell count.

Neutrophilia is a high neutrophile count.

Lymphocytosis is a high lymphocyte count.

Monocytosis is a high monocyte count.

Eosinophilia is a high eosinophile count.

Hyperleukocytosis is a leukocytosis over 25,000.

Leukopenia is a low white blood cell count.

Neutropenia is a low neutrophile count.

Lymphocytopenia or **lymphopenia** is a low lymphocyte count.

Monocytopenia or **monopenia** is a low monocyte count.

Hypoeosinophilia or **eosinopenia** is a low eosinophile count.

Toxic granules are large, irregularly clumped granules seen in the cytoplasm of the neutrophils in disease processes such as severe bacterial invasion and chemical intoxication.

The **hemogram** is a complete picture of the blood, including numerical and differential counts, hemoglobin estimations, blood platelet counts, reticulocyte counts, special tests, and complete examination of the stained erythrocytes.

The **hemogram of Schilling** is a linear depiction of the differential count of leukocytes, so arranged as to show at a glance a pathologic shift in the differential picture.

A **nuclear shift** is an increase or decrease in the number of neutrophilic cells of one type over another. (See page 779 for detailed description.)

A **degenerative leukocytic blood picture** is one which shows an increase in the number of "stab" cells over normal (together with other findings), due to a toxic depressing influence on the hematopoietic organs.

A **regenerative leukocytic blood picture** is one which shows an increase in the number of "juvenile" or "metamyelocyte" cells over normal, or an occasional myelocyte, usually with an increased white blood cell count, due to stimulation of leukopoiesis.

A **leukemoid** or "liberation" or "flooding" leukocytic blood picture is one which shows a marked increase in the number of myelocytes, juveniles, and "stabs" over normal, signifying total loss of defense of the hematopoietic organs. Nucleated red blood cells may be seen. This picture must be differentiated from leukemia.

Leukemia is a fatal disease of unknown origin, which primarily involves white blood cells, which may or may not be increased in numbers in the blood stream; pathologic forms of leukocytes, often very young, are always present in this disease. (See page 845 for further elaboration.)

Blood platelets (thrombocytes) are small bodies found in the circulating blood, which bear an important relationship to blood clotting.

Thrombocytosis is a high blood platelet count.

Thrombopenia or **thrombocytopenia** is a low blood platelet count.

Hyperplasia is an overgrowth of tissue. In reference to blood formation, this may refer to any or all of the blood elements. It is most strikingly seen in the leukemias.

Hypoplasia is diminished tissue formation.

Aplasia is cessation of tissue formation.

Blood dust, or **hemokoniae**, are small refractive bodies in the blood, supposed to be fragments of the red blood corpuscles.

Chemotaxis is the phenomenon shown by certain living cells of moving toward (positive) or away from (negative) certain other cells or substances which exert a chemical influence.

Agglomeration is the adhering together of cells of the same kind; such as, rouleaux formation of erythrocytes, groups of white cells, thrombus formations of blood platelets, heaping up of eosinophiles, etc. This process is important in sedimentation tests, and in the making of blood spreads.

Mitosis is indirect cell division, through nuclear changes, or **karyokinesis**. It is the typical mode of division of active somatic cells and germ cells.

HEMATOPOIETIC ORGANS AND THEIR PHYSIOLOGY

Hematopoiesis is a function of many organs acting and reacting in a complex manner. The adult circulating blood is a definite resultant of these activities. The blood-forming organs constantly manufacture blood as a phase of physiologic regeneration. The blood cells are destroyed peripherally and in certain organs by emigration into and consumption in the tissues. We are interested in the study of blood formation, of blood consumption, and of blood

regeneration, and in the reaction of blood in the presence of disease. Changes in the red cells occur in the so-called anemic diseases; changes in the white cells are most important in the course of infection. The changes in cells may be in quantity (decrease or increase in total number) and in structure (either in the appearance in the circulating blood of cells found normally only in the hematopoietic organs or in the appearance of pathologic forms not otherwise seen in the circulating blood).

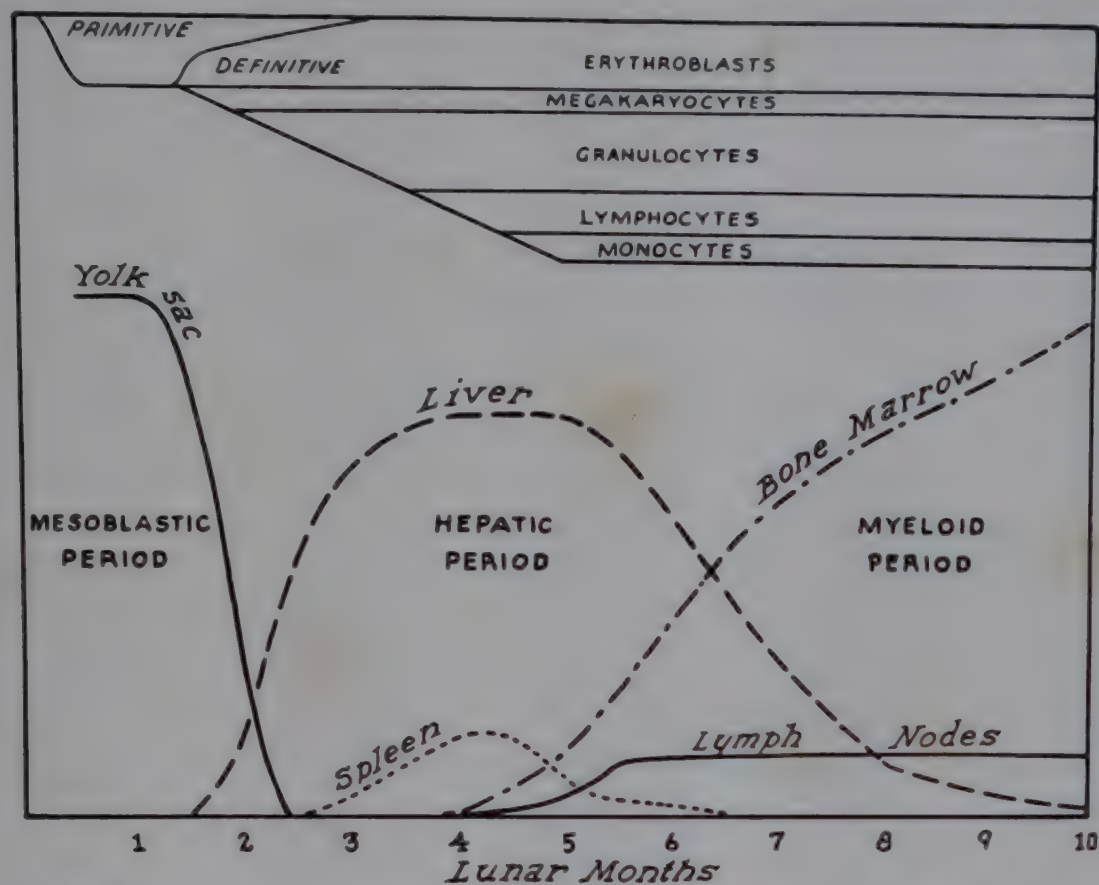


Fig. 138.—Stages of hemopoiesis in the embryo and fetus, indicating the comparative participation of the chief centers of hemopoiesis and the approximate times at which the different types of cells make their appearance. (From Wintrobe, M. M.: Clinical Hematology. Lea & Febiger, 1952. Reproduced by permission of the author and the publisher.)

Embryonal Blood Development.—

Blood cell formation takes place primarily within the body in the embryonal state in the area vasculosa. From this emanate angioblasts. The nomenclature in this stage of embryonal blood development is still in controversy. Minot terms the first intravasal cells "mesamoeboid" cells and believes them to be the parent cells of all blood cells (Pappenheim, Maximow, Dantschakoff, Naegeli, Schridde). These cells are transformed into erythroblasts by the formation of hemoglobin; in the human embryo, this occurs at the 8 mm. stage of development. Sabin noted a very early genetic separation of granulocytic, monocytic, and lymphocytic centers. She believed that granulocytes came into existence from the direct transformation of angioblasts, and that lymphocytes originated from body cells at a considerably later period than the other cells just described. As noted before, Maximow considered the small lymphocytes the parent cells of all blood cells, while Naegeli drew a very sharp line between myeloblasts and lymphoblasts.

It seems undeniable that the potential differentiation of cells precedes the formation and development of their specific organs, thus proving a difference in the physiologic function of each cell system. The Schilling idea assumes that an embryonal cell evolves polyphyletically in various directions, but that it is impossible for a differentiated myeloblast to develop into either a lymphocyte, monocyte, or erythroblast. The first localization of hematopoiesis may be observed in the liver of the 12 mm. embryo, which is in the third month of embryonal life. At this time there begins the development of the outstanding blood organ, namely, the bone marrow. While it is true that the differentiation of cells occurs in early embryonal stages, physiologic regeneration of injured or lost blood cells later in life suggests the possibility of later new differentiation of various cell types from certain elements that remain indifferent and latent, that is, mesenchymatous or angioblastic cells.

Blood is formed by mitotic and amitotic division of the younger cells. The youngest cells are differentiated into nuclear forms, protoplasmic structures, or histologic arrangements. Blood cells develop in close proximity extracapillarily to the vessels and intracapillarily to the erythrocytes. We believe that a pathologic extramedullary hematopoiesis, such as is seen in toxic and tumor processes, is purely a regenerative phenomenon. Oeller has cited the local production of free blood cells in inflammatory conditions in connective tissues. Maximow believes in the transition of endothelial cells into fibroblasts, and of fibroblasts into phagocytes and monocytes but not into other blood cells. Most writers agree that erythrocytes, mature lymphocytes, and granulocytes perish in the end in a uniform manner.

The Schilling concept is the direct reverse of the Maximow unitarian idea. Phylogenetic studies indicate that there is a definitely well-developed differentiation of all blood cells. Tendencies in the development of the growing organism determine the local situation of the cells; viz., myelocytic cells in the bone marrow, lymphocytic cells in the region of the lymph vessels, and monocytes in the reticuloendothelial structures.

Bone marrow formation begins in the third embryonal month and soon changes into myelocytic elements. Transformation of connective tissue cells into lymphoid "wandering cells" soon ceases, and differentiated tissue—erythroblasts, myeloblasts, giant cells—appears. Erythroblastic and megaloblastic cells soon are seen. Granulocytic elements are scarce at first and are isolated or in small groups. The differentiation of basophilic and eosinophilic cells in the bone marrow is a very early phenomenon. Embryonal bone marrow is very rich in cells and remains so in the first years of life, gradually changing as infancy begins. It is to be noted that the bone marrow of the long bones in the embryos and children is more hematopoietic than that of adults. Our attention will later be called to the fact that bone marrow may be changed into fat and may change back again into red marrow. Red marrow may be found in all bones of the newborn, decreasing in the child up to the end of the second year. Gradual concentration of the marrow into the smaller bones occurs and red marrow is seen in adult life only in the proximal epiphyses of the long hollow bones.

In the human after birth, hematopoiesis is concentrated in the bone marrow and the lymph nodes. Normal bone marrow is the primary source of erythrocytes, cells of the myelocytic series, and platelets. Lymph nodes are the major source of lymphocytes, with the spleen contributing to the production of these cells.

General Facts on Cellular Development.—

All cells arise from pre-existing cells by division. This fact was first brought out in the celebrated dictum of Rudolf Virchow—“*omnis cellula e cellula.*” Cells divide in two ways—by amitosis and by mitosis. Amitosis is

SUCCESSIVE STAGES OF MITOSIS

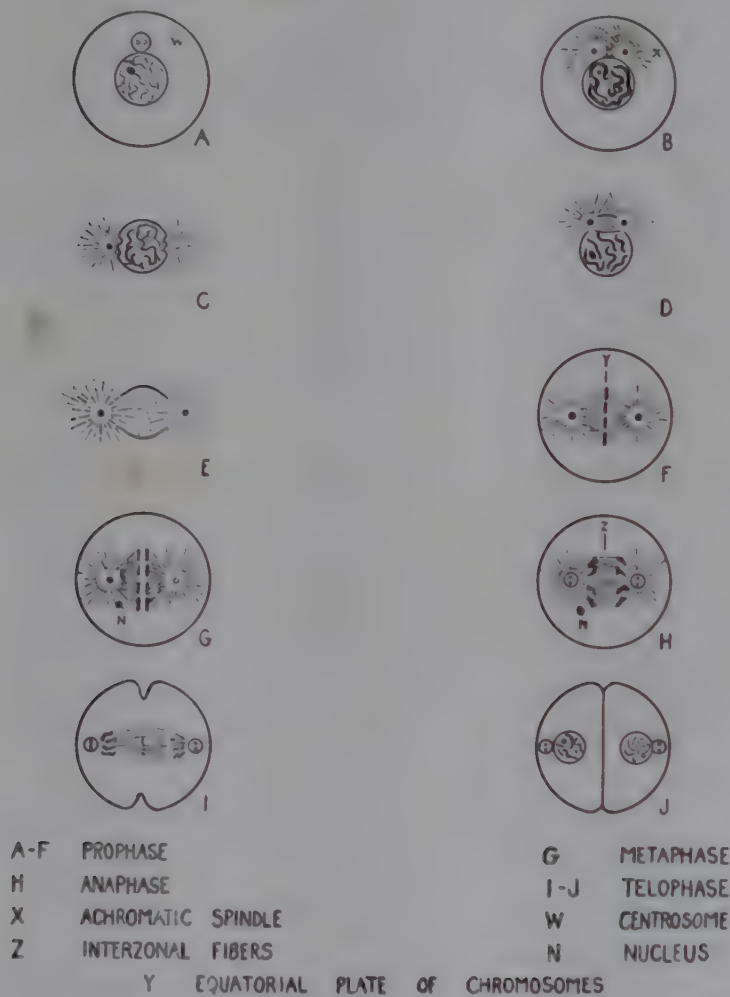


Fig. 139.—Successive stages of mitosis. (After Wilson.)

direct division of cells by the simple fission of nucleus and cytoplasm. It is not preceded by structural metamorphosis of the nucleus or cytoplasm. It is seen constantly in the cells of the blood and lymph, the osseous medulla, the bladder, epididymis, liver, placenta, cartilage, tendons, cells of Sertoli, mesenchyme, etc. In mitosis, in the multiplication of typically active somatic cells, and in all germ cells, complicated changes take place in the nucleus. Since these changes give rise to threadlike structures developed out of the nuclear chromatin, the process is termed mitosis from the word “*thread*,” in contrast to amitosis, or “*no thread*.” The distinctive feature of mitosis is the production of a characteristic number of chromosome bodies, their accurate lengthwise halving, and the equal distribution of these halves to the daughter cells.

Mitosis or *karyokinesis* was first discovered by Schleicher and studied by Flemming. It is the most important method of cell division. There are different types of indirect generation. The phases of the process, beginning with the state of rest, are classified as progressive and regressive. The progressive changes of structure occur between rest and the phase of metakinesis or mother star. The regressive retrocede from the height of metakinesis to the resting stage of the daughter cells. The order and nomenclature of the phases are as follows:

First, the *progressive phases* occur. These are divided into the *prophase* and the *metaphase*. In the prophase stage there are first, the phase of rest; second, the phase of gross segmentation; and third, the phase of the ovum. In the metaphase stage there are the phase of transverse segmentation and the phase of the mother star, or metakinesis. *Prophase*.—The centrioles separate from each other with the formation of attraction spheres around each of them while the chromatic masses change into *chromosomes* whose number is constant in a given species (48 in man). *Metaphase*.—Each chromosome splits into two equal parts.

There are two states in the regressive phases, the *anaphase* and the *telophase*. *Anaphase*.—The halves of a particular chromosome separate and each half approaches one of the two centrioles which have migrated in the meantime to opposite poles of the cell. *Telophase*.—The formation of the two daughter nuclei and the division of the cytoplasm into masses surrounding each daughter nucleus takes place.

In the *prophase* state, the chromatic net of the nucleus becomes richer and the nucleus increases in size. The idiozome and the centrioles become visible. Reticulation occurs preparatory to the organization of the ovum.

In the *phase of the ovum*, there are seen two classes of trabeculae, primary or gross, formed principally from the chromatin; and, secondary or fine, constituted by the linin threads. The primary filaments, or chromosomes, are disposed in hairpin-like forms orientated almost always in the same direction with the elbows turned to one side. The site of the nucleus where the angles of the hairpins converge presents a space devoid of filaments, the polar field. The opposite extreme, antipode or contrapolar field, is filled by the accumulation and intercrossing of the free ends of the chromosomes.

At the end of the *spireme stage* the chromatic filament divides into the number of chromosomes characteristic of the species of the cell. These chromosomes assume the form of hairpins.

During the *metaphase*, or in metakinesis, the chromatic threads or hairpins are arranged in the form of a star. Another characteristic of this phase is the enlargement and dislocation of the achromatic spindle by the division of the chromosomes. The chromosomes dispose themselves around the widest region of the spindle in the form of a star, at whose perpendicular or equatorial plane occurs the segmentation of the nucleus and protoplasm.

In the *anaphase*, the chromosome hairpins are duplicated and begin to separate from the elbows toward the extremities. There is now seen a running of the achromatic handles toward the poles forming a double star or diaster.

In the *telophase*, the centrosomes and the spindle disappear while a fine membrane forms around the daughter chromosomes. The nucleus takes the form of a horseshoe. This protoplasmic furrow reaches the equator of the spindle and

forms a thin bundle of very fine threads. Shortly after this, the bundle bursts and the intermediary body disappears. According to Flemming, the nuclei of the human cells contain approximately 24 pairs of chromosomes.

In direct, or *amitotic* division, the nucleus is thought to constrict and finally to divide into two halves, a nucleolus going to each daughter nucleus. There is much discussion as to whether this process actually occurs under normal conditions. Although direct observation of living cells has shown that a nucleus may constrict into two apparently completely separated daughter nuclei, there are no well-authenticated instances of accompanying complete separation of the cytoplasm. It is claimed that if a cell once undergoes amitotic division it is no longer capable of going through indirect (mitotic) division.

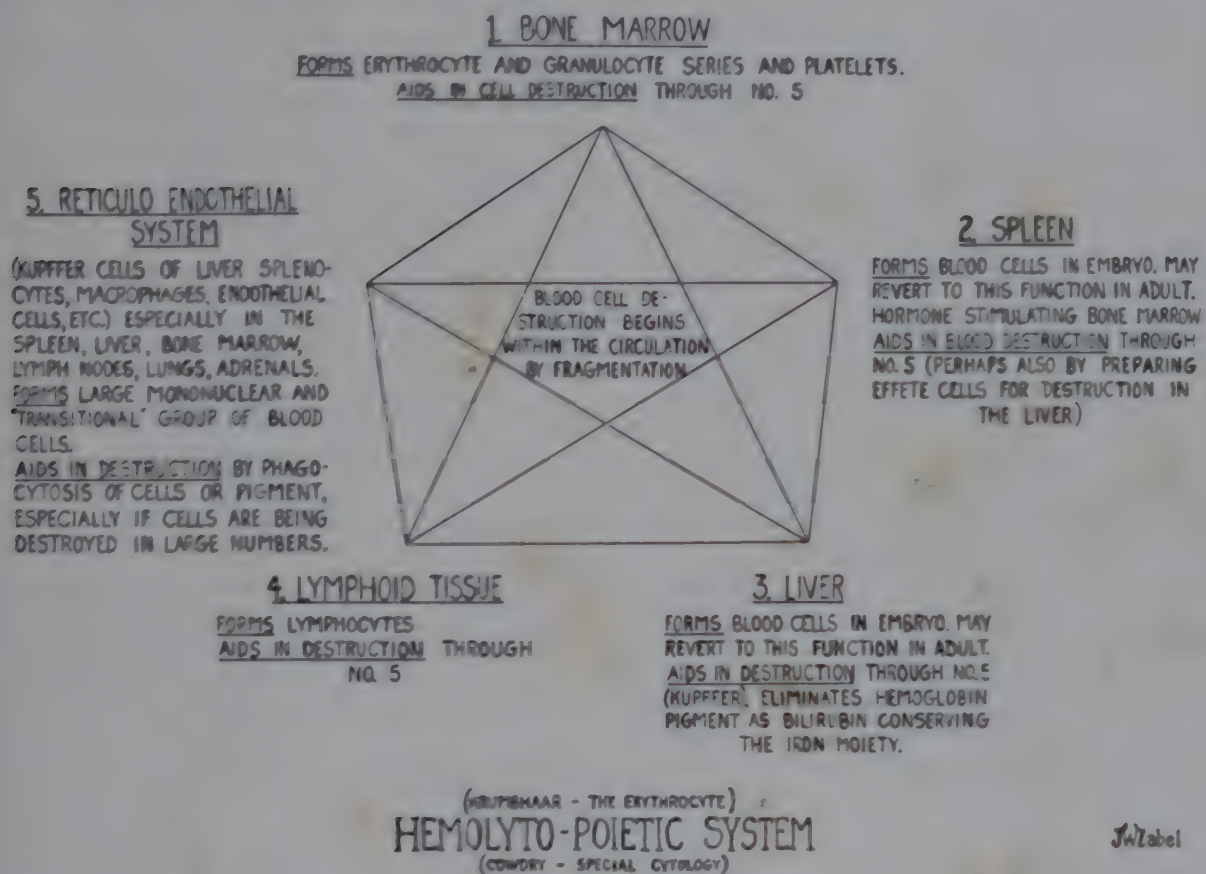


Fig. 140.—Maximow's Chart.

Bone Marrow.—Normal bone marrow shows different types of cells. Types commonly found are the mature neutrophils, juvenile forms, and "stab" neutrophils, as well as basophils and eosinophils. Lymphocytic cells are normally present in bone marrow, presumably entering by way of the blood stream or occasionally being normally formed in the marrow. Plasma cells are also seen in marrow but their origin is uncertain. In normal bone marrow are seen pro-erythroblasts but the more common cells are the erythroblast and normoblast. The erythroblast develops from the pro-erythroblast by a condensation of the nuclear chromatin and a loss of the nucleoli. The normoblast develops from this cell by further condensation of the nuclear chromatin and the appearance of hemoglobin in the cytoplasm. Megakaryocytes are present in normal bone marrow and are generally believed to be the source of blood platelets (Wright).

Refer to pages 914 ff. for a complete discussion.

The Fate of Red and White Blood Cells.—Attempts are being made constantly to determine the daily blood cell production. So far as the red blood cells are concerned, the amount of bilirubin produced from hemoglobin over hematin and hematin may serve as a basis for calculation. Some writers state that the life of the red cell is from 20 to 53 days. Many different methods have been used to estimate the average life span of the human red blood cell. The different attempts have yielded estimates which range from 5 to 200 days. Determinations of the amount of iron or pigment excreted have yielded estimates of 20 to 200 days.¹ Determinations of the survival time of transfused red cells by means of differential agglutination yielded estimates ranging from 30 to 100 days. The use of agglutinogens M and N for tagging red cells gave values of 80 to 120 days. Callender, Powell, and Witts,² using Ashby's differential agglutination method³ in Rh-positive men,⁴ concluded from a mathematical analysis of their data that red blood cells live for approximately 120 days. Measurement of the time required for the disappearance of sulfhemoglobin from the blood of cyanosed workers⁵ indicated the life span of the red blood cells to be 115 days. Shemin and Rittenberg⁶ reported their results in estimating the life span of the red blood cell by estimating isotope concentration of the heme at various periods in experimental works. They showed that the heme of the nonnucleated red blood cells, unlike the constituents of nucleated cells, is not continuously formed and degraded within the cell. The red cells are not indiscriminately destroyed, but their rate of destruction is a function of the age of the cell. Study of the isotope concentrations found in the heme of the human red blood cell, after the feeding of glycine labeled with N¹⁵, indicated that the erythrocyte is not subjected to indiscriminate destruction but has a life span. This was found to be about 127 days.

Assuming that the life span is 120 days, and estimating 5,000,000 red cells per cubic millimeter of blood, the bone marrow would have to manufacture about 250 billions of red cells every day. This means a production of 42,500 red cells for each cubic millimeter of blood each day. About 0.83 per cent of the red cells and hemoglobin are replaced daily.

At this time neither the duration of life nor the mode of destruction of the white cells is definitely known. It is generally believed that disintegration of leukocytes in the blood stream does occur. It is probable that white cells are consumed in greater amounts than the red cells. Since there are only one-thousandth as many white cells in the peripheral blood as red cells, the time of their development must be longer or their life must be much shorter. There must be, according to the present figures, at least an age of two hours for a leukocyte. Ponder, on the basis of Arneth's shift to the left, attempted to calculate the life of leukocytes and found it to be from two to three weeks. The segmentation of white cells, according to Schilling, is not a measure of their age. Magner⁷ states that the life of the leukocytes is probably not longer than a few days, but that some workers believe that the nonmotile cells which

¹Schidt, E.: *Acta med. scandinav.* 95: 49, 1938.

²Callender, S. T., Powell, E. O., and Witts, L. J.: *J. Path. & Bact.* 57: 129, 1945.

³Ashby, W.: *J. Exper. Med.* 20: 267, 1919; 34: 127, 1921.

⁴Landsteiner, K., and Wiener, A. S.: *Proc. Soc. Exper. Biol. & Med.* 43: 223, 1940.

⁵Editorial, reference to Jope, unpublished work, *Brit. M. J.* 1, 576, 1946.

⁶Shemin, D., and Rittenberg, D.: *J. Biol. Chem.* 166: 2, 627-635, Dec., 1946.

⁷Magner, Wm.: *Hematology*, Philadelphia, 1937, P. Blakiston's Son & Co., Inc., p. 77.

are seen in supravitally stained preparations are artefacts. Many leukocytes penetrate the capillary walls, wander through the tissues or escape from the body through mucous surfaces, but there is no evidence that the neutrophilic leukocytes exercise any function under normal conditions. By virtue of their phagocytic powers, and by the production of bacteriolytic substances, they are active in the defense of the body against pyogenic bacteria and they also play an important part in the digestion of necrotic tissues by means of proteolytic enzymes.

Returning to the question of the origin of erythrocytes, according to the Schilling idea, the formation of these cells begins as indifferent erythrogonia, followed by the proerythroblast with nucleoli, then by typical, hemoglobin-containing, macrocytic erythroblasts, then polychromatic and orthochromatic, smaller normoblasts. Then, in turn, come the denucleated polychromatic cells, and, finally, the full-grown orthochromatic normocytes. There are many explanations of the denucleation of the erythrocyte. First, Maximow claimed that the expulsion is seen in the embryonic body although this is denied by Schridde. Albrecht is in favor of the expulsion theory. A second theory is that of karyorrhexis, where the pyknotic nucleus assumes bizarre shapes and is dissolved into spherical sections, leaving the cell gradually. Weidenreich² maintained that karyorrhexis may go on to further reduction, giving the appearance of small chromatin rodlike changes. Next, there is a theory of intracellular nuclear disintegration, which is largely the belief of Naegeli. Schilling's theory of the origin of blood platelets is in line with the denucleation idea. This, of course, as indicated in another section in this work on blood platelets, is opposed by a number of writers. Hittmair³ showed that blood platelets appeared in the embryo at a stage when there are only a few leukocytes in the blood. The Wright theory,⁴ in which the majority of American hematologists concur, represents blood platelets as remains of broken-off pseudopodia of the cytoplasm of megakaryocytes. Naegeli agrees with this view and supports it with the statement that blood platelets appear in the embryo simultaneously with the megakaryocyte and both forms are found in all mammals but not in vertebrates of other species. The work of Pianese and Watson has already been touched upon. (See remarks on Blood Platelets, pages 554 and 559.)

It is interesting to note the remarkable amount of basophilic substance in the young forms of the erythrocytes and also to note when hemoglobin is first seen in embryonic blood. Wulf claims to have seen traces of hemoglobin by means of the spectroscope in the embryo with nine primary segments. The hemoglobin shows up first in the mitochondria, which are found in the youngest cell. In this connection, study of polychromasia by supravital staining methods shows that it may degenerate into basophilic punctation. In other words, polychromatic vital-staining reticular substance and basophilic punctation are protoplasmic and not nuclear phenomena. Normally, polychromasia is seen in peripheral blood in the proportion of 0.2 to 1.0 per cent of all red cells. Pathologically, this may range as high as 50 per cent, as seen in the

²Weidenreich, E. R. G.: *Anat.* 14: 15, 1905.

³Hittmair, A.: *Folia haemat.* 35: 156, 1927.

⁴Wright, J. H.: *Boston M. & S. J.* 154: 643, 1906.

hemorrhagic anemias, blood intoxications, and aftereffects of acute infections, malaria, tuberculosis, syphilis, in blood crises of pernicious anemia, and in hemolytic icterus.

Physiologic processes which may affect erythropoiesis are menstruation and pregnancy, hemorrhage, and blood destruction. There is a rapid production of red cells after venesection, first, in the form of migrating maturing forms, and second, as nucleated elements, immature polychromatic erythroblasts and normoblasts. At this time, liberation of many young forms takes place. Any decrease in the quantity of erythrocytes is regarded as a physiologic stimulant to the formation of more erythrocytes. However, daily blood transfusions in quantity equal to the destroyed blood lead to inertia of the destroyed bone marrow and aplasia. A single injection of a physiologically destroyed amount of erythrocytes results in immediate irritation of the bone marrow. This irritation may be followed within 4 to 7 days by a prolonged increased erythropoiesis. Lack of oxygen may also be an effective physiologic stimulant, such as is seen in high altitudes. Increase in the number of erythrocytes due to chronic lack of oxygen is seen in heart diseases, congenital pulmonary stenoses, mediastinal tumors, etc. (see pages 769 ff.). A great many of our ideas concerning regeneration of red blood cells have been changed since the advent of liver therapy in the treatment of pernicious anemia. This will be alluded to in our remarks under pernicious anemia.

The physiology of the formation of granulocytes has been thoroughly studied. We know that granulocytes, like erythroblasts, are formed in well-defined centers, and that promyelocytes and myelocytes, characterized by blue cytoplasm and light, nucleolated nuclei, develop from these centers. In the bone marrow, before this stage has arrived in the maturation of granulocytes, there can be seen large, basophilic cells with nucleolated nuclei, the so-called Pappenheim's "lymphoidocytes," Maximow's "polyblasts," or primitive blood cells, Ferrata's "hemocytoblasts" or Schilling's "undifferentiated" parent cells. Table 46, from Schilling,¹ illustrates the various stages through which the granulocyte passes before it becomes a fully mature segmented granulocyte.

In this connection, attention must be called to the differences between the Arneth and Schilling theories of shifts in blood pictures, which is discussed under our remarks on differential counts. Arneth's ideas represent a theory of consumption and substitution, whereas Schilling's belief is a concept of regeneration or accelerated new formation of granulocytes, and degeneration of the blood manufacturing centers.

The Normal Rhythm of the White Blood Cells.—Leukocytic life is rhythmically regulated. The contribution of Sabin, Cunningham, Doan, and Kindwall² covers the question of the normal rhythm of the white blood cells. It seems that from hour to hour and from day to day the number of white cells remains remarkably constant. This contribution has given us a better understanding of the physiology of the white blood cells. The study was worked

¹Schilling, V.: *Handbuch der normalen und pathologischen Physiologie*, Part 2, Berlin, 1928, Verlag von Julius Springer, p. 789.

²Sabin, F. R., Cunningham, R. S., Doan, C. A., and Kindwall, J. A.: *Bull. Johns Hopkins Hosp.* 37: 14, 1925.

TABLE 46

Nucleus	Cytoplasm
<i>Myeloblast</i> (Naegeli): round, finely reticular in structure, with blue plastin nucleoli.	Highly basophilic, no granules; faint to strongly positive oxydase reaction.
<i>Promyelocyte</i> (Pappenheim): round, with a tendency to polymorphism and lobulation. This is especially true when these cells are seen under pathologic conditions. Plastin nucleoli are at times faint or completely absent. The whole structure is denser and darker.	Dark blue to pale blue. Beginning deep red azurophilic finer or coarser granules (progranules).
<i>Myelocyte</i> (Ehrlich): round, oval, to slightly indented, only chromatic nucleoli; coarser and darker interstices.	Pale blue to neutral, mature neutrophilic, basophilic, or eosinophilic granules.
<i>Juvenile granulocyte</i> (Schilling): sausage-shaped, well-filled structure distinctly divided into fields, frequently with polar nucleoli.	Occasionally slightly basophilic, usually neutrophilic granules very intensely stained, with a tendency to azurophilic characteristics.
<i>Stab or rod nuclear granulocyte</i> (Schilling): slender, darker, often with a twisted nucleus with bizarre indentations suggestive of beginning segmentation.	Mature granules.
<i>Segmented granulocyte</i> (Schilling): nucleus has many divisions, two to five segments connected in a chain by fine threads or bridges.	Mature granules.

out through supravital technic. As described by Sabin,* a type of cell exists in the blood which she called the "nonmotile leukocyte." This loss of movement is essentially a chemical and physical change in the neutrophilic granules, together with a loss of power to react to vital stains. This cell was first described by Schilling¹ in 1908. He noted the cessation of motility and also described the subsequent disappearance of the centrosphere. He maintained at that time that it represented the physiologic death of the leukocyte. Cunningham, Sabin, and Doan noted the showerlike appearance of these dying cells in the blood. They believed that if one could arrive at an estimate of the amount of cell death of the leukocytes in the blood stream one might devise some method of analyzing the rhythm of the leukocytes. Counts of the white cells of human subjects at different times of the day had previously been made. Torday² found the lowest count in 61 cases was 3,130 and the highest 9,800. Turk³ found the normal count at different times of day was from 5,000 to 10,000 white cells. Most of the studies on the rise and fall of leukocytes have been made on pathologic cases. Investigators have found that the injection of foreign proteins, bacteria, drugs, etc., produces a very definite leukopenia followed by a leukocytosis. It has been found by some observers that variations in the blood pressure are followed by changes in the number of blood cells, a lowering in the pressure being followed by a decrease in the number of cells.

*Sabin, F. R.: Bull. Johns Hopkins Hosp. 34: 277-288, 1923.

¹Schilling, V.: Folia haemat. 4: 429, 1908.

²Torday, A. V.: Virchows Arch. 191, ccxiii, 529-536.

³Turk, W.: Wien, 1912, W. Braumüller, II.

Arneth⁴ and others have found that there is a definite increase in the number of white blood cells after cold baths. Change in posture has been followed by variation in the number of leukocytes. It was also found that muscular work caused a constant increase of leukocytes and that this disappeared in the course of 2 to 3 minutes, but reappeared again on renewed exercise.

Digestive leukocytosis has been studied by a number of investigators. Pohl⁵ studied digestive leukocytosis in dogs after 18 hours of fasting and found after feeding them meat that they had a leukocytosis beginning in one hour and reaching a maximum at three hours. With nonalbuminous foods he obtained no leukocytosis. Rieder⁶ confirmed Pohl's findings both in man and in dogs. Sirensklj,⁷ in studies on man, found a digestive leukocytosis after meals rich in albumin or in fat, but only a very slight reaction after food consisting of carbohydrates alone. Pirone⁸ from histologic studies of the blood-forming organs during digestion concluded that the reaction had three phases: (1) increased destruction of the older blood cells; (2) increased stimulation to the formation of new cells and the migration of elements into the blood stream; and (3) that the bone marrow, spleen, and lymph glands all showed signs of hyperfunction and increased hematopoietic activity. The work of Cunningham, Sabin, and Doan has given us the best information on the question of the rhythm of white cells. It is fairly constant for 7 to 8 hours as shown by supravital preparations. They showed that the white blood counts are higher in the afternoon than in the morning quite independently of whether food is taken. They studied supravital-stained specimens as well as fixed films and found that neutrophilic leukocytes of the supravital counts are 6.3 per cent higher than in the differentials from the fixed films, but the total leukocytes are only 2.3 per cent higher. The lymphocytes were found in practically the same amount; namely, 3.5 per cent lower. The basophiles and eosinophiles are higher in the supravital counts than in the fixed films. With respect to the monocytes, the conditions are reversed, for their percentages are slightly lower in the supravital technic than in the fixed film.

Physiology of the Lymphocytes.—According to Sabin,⁹ lymph sacs are formed in the embryo of 6 to 24 mm. near the jugular vein. These sacs become the so-called lymph hearts. The latter form the primary lymph glands (embryo from 10.5 to 30 mm.). The first lymph follicles appear in the embryo of 50 mm. Lymphocytic cells develop from the mesenchymatous migratory cells of the adventitia of blood vessels in the tissues between the lymphatic capillaries. Maximow¹⁰ designated the first free mesenchymatous migratory cells as lymphocytes. He insisted that from the primitive cells true small lymphocytes may form, directly or indirectly, and that even the lymphocyte of the blood preserves this polyblastic embryonal capacity.

The follicle is the unit of true lymphatic tissue. Lymphatic tissue is found extensively all over the body. The glands and spleen furnish the true lympho-

⁴Arneth, J.: Leipzig, 1920, W. Klinkhardt.

⁵Pohl, Julius: Arch. f. exper. Path. u. Pharm. 25: 51-68, 1889.

⁶Rieder: Leipzig, 1892.

⁷Sirensklj, N. N.: Dissert., St. Petersburg, 1908.

⁸Pirone, R.: Sperimentale 61: 83-94, 1907.

⁹Sabin: Ergebn. d. Anat. 21: 1913.

¹⁰Maximow: Folia haemat. 8: 125, 1909; Ann. anat. path. 4: 1927; v. Mollendorfs Handb. d. mikrosk. Anat. 2: 1927 (Literatur).

cytes to the blood stream. The follicle has a germinating center. This consists of lighter and larger cells with vesicular nuclei and distinct nucleoli. Follicles are very rare in a fetus. The follicle consists of delicate fibers and reticular cells. Its capillary system is radially arranged. The interfollicular tissue is made up of a net of reticular cells and free macrophages, which together with numerous lymphocytes fill the meshes. Flemming believed that the formation of lymphocytes emanates from the cells of the germinating centers, while Naegeli and Schridde call these cells lymphoblasts. While Schilling observed that true lymphocytic cells have been seen in the bone marrow, especially in chronic conditions, he believes with the dualists that these cells do not belong with true bone marrow tissue. In extreme conditions of lymphocytic leukemia, the parenchyma of the bone marrow is virtually suffocated by the densely proliferating lymph centers. According to the Schilling viewpoint, true lymphocytes come from the lymphatic parent cells in the centers in lymphatic tissue. Increase of lymph cells in the periphery is considered an expression of increased lymph cell formation. We know that about one-fourth of the circulating cells are lymphocytes, but they are only a small part of the total number manufactured. Most of the blood lymphocytes are brought forth by the spleen and glands.

The function of lymphocytes is recognized in the walling off of chronic inflammatory foci. They are increased in chronic inflammatory states. They are increased during infancy and in recovery from acute infections and in certain diseases such as measles, mumps, whooping cough, undulant fever. In tuberculosis there is an increase in lymphocytes when the process is being arrested. They are increased in infectious mononucleosis and in acute and chronic lymphocytic leukemia. The lymphoblast is seen in acute lymphocytic leukemia and in children showing lymphoid hyperplasia. See pages 643, 851 ff., 856.

Physiology of the Monocytes.—Ehrlich called all cells with excentric pale nuclei, large, faintly basophilic cytoplasm, generally without granulations, "large mononuclears." He differentiated these from "transitionals" which had deeply indented nuclei, constituting the beginning of neutrophilic granulations. He believed that these cells were transitions to neutrophils. Pappenheim claimed that they were older lymphatic forms of the Rieder type. All authors except Pappenheim traced these cells to the neutrophilic system. Einhorn and Ehrlich believed that a relationship existed between these cells and the spleen and called them "splenocytes." Pappenheim and Ferrata in 1910 finally suggested the name "monocytes" for both the mononuclears and transitionals. Pappenheim¹ was the first to identify the monocytic granules as *not* neutrophilic and *not* true granules, and Naegeli admitted in the same year that the granules were azurophilic and not neutrophilic. Schilling emphasized that the oxydase reaction indicates that the monocytes occupy something like an intermediate position between the other systems. As noted in our remarks on monocytic leukemia (pages 857 ff.), Schilling and Reschad reported the first case of true leukemia with basophilic young monocytes, an average number of monocytes of 74 per cent, and total leukocytes up to

¹Pappenheim: *Folia haemat.* 12: 1912.

56,000. The bone marrow and lymphatic tissue were unchanged histologically. Schilling demonstrated basophilic juvenile forms in variola and also the close relationship between the blood monocytes and endocarditis lenta. He concluded that the peripheral macrophage is merely a functional form, coming from the same parent cells as the monocytes, and that the monocytes could be converted into macrophages.

Schilling sought to find the genesis of monocytes in the reticuloendothelium structure, that is, the endothelium of hematopoietic organs and the Kupffer cells of the liver. Maximow opposed this view with his histiocytic or lymphocytic origin of monocytes. He explained the monocytes as "physiologic blood polyblasts."

The Functions of the Spleen.—

The general conception of the function of the spleen is that it is a reservoir or bank of blood. In fetal life erythropoiesis begins in the spleen but the main function of the spleen is that of lymphopoiesis. The spleen is concerned with the destruction of "worn-out" red cells and blood platelets and also assists in the destruction of protozoa and foreign material. In fetal life myeloid cells are also produced in the spleen. The spleen also plays an important role in the conservation of iron derived from broken-down corpuscles. This organ contains more endothelial tissue than any other organ of the body. In adult life lymphocytes are produced by the malpighian corpuscles. Many believe that monocytes may arise in the spleen largely because of its abundant reticuloendothelial structure. In severe chronic anemias there are foci of extramedullary blood formation. It is also probable that the spleen exercises some sort of control over the bone marrow. Splenectomy is often followed by the appearance in the peripheral blood of nucleated red cells, Howell-Jolly bodies, basophilic cells and reticulocytes, together with an increase in the number of platelets. Following splenectomy, there is also a great increase in the number of leukocytes. It is the appearance of Howell-Jolly bodies, however, that is the most characteristic phenomenon following splenectomy.

The unusual and abnormal red corpuscles seen in the blood after splenectomy is held by some to mean that the spleen has a definite effect on the maturation of red cells.

The Functions of the Blood.—

The chief functions of the blood are: to transport food materials to the tissues; to carry oxygen from the lungs to the tissues and carbon dioxide from the tissues to the lungs; to carry waste materials from the tissues to the excretory organs; to transport endocrine secretions from one organ to another; to act as a defense mechanism against infection through the activities of certain white cells and immune bodies developed in the blood stream; to assist in maintaining a constant water balance and correct osmotic equilibrium in the tissue cells; further, to assist in the maintenance of a constant body temperature and to maintain a degree of irritability of the tissue cells so that functional activity may be carried on satisfactorily.

IMMUNOHEMATOLOGY

Immunohematology, a term proposed by Chauffard and Troisier¹ in 1908, in recent years has attracted the attention of clinicians and investigators. The discovery of the mechanisms involved in Rh sensitization perhaps gave this field its greatest stimulus, and today the literature is replete with references to immunologic mechanisms as operative in many blood dyscrasias. Although antigen-antibody relationships have been well established for iso-immune phenomena related to red cells, knowledge of the phenomena of autoimmunity is of relatively recent origin.² Evans and his co-workers³ suggested a common etiology of immunologic character for thrombocytopenia and acquired hemolytic anemia; Harrington and his group⁴ brilliantly completed the picture as related to thrombocytopenia; and Moeschlin and Wagner⁵ have added the leukocyte to the immunologic fold. The rapidly expanding literature in the field of immunohematology contains numerous studies on clinical states in which one of the formed blood elements is predominantly involved or combinations of two or more of the elements are affected. In the consideration of hemolytic anemias, thrombocytopenias, or leukopenias, an immunologic mechanism must be considered. The field is much too extensive to cover completely; some of the high spots will be touched upon briefly.

In the development of immunohematology the classic approaches of the immunologist have been used, with modifications in methods as new problems have presented themselves. These have consisted of (a) finding antibodies in patients with hematologic disorders; (b) in vitro demonstration of such antibodies; (c) in vivo demonstration of antigen-antibody relationships; and (d) experimental production in animals of antibodies against specific blood elements.

These approaches have been most fully developed in relation to the red blood cells as exemplified in the hemolytic anemias. Isoimmunization as related to the blood groups is a well-established field, becoming more complex only as new blood factors are uncovered. In such sensitization, incomplete antibodies in the form of "agglutinins" may be demonstrated either by testing trypsinated red cells or by the antiglobulin test (Coombs' direct or indirect). It is in the field of the idiopathic acquired hemolytic anemias that the picture is less distinct; in only about 80 per cent of these cases, incomplete agglutinins may be demonstrated. In the hemolytic anemias associated with intracorpusecular defects (thalassemia, hereditary spherocytosis, hereditary ovalocytosis, and molecular disease of hemoglobin), these antibodies are only rarely demonstrable. Other serologic findings in autoimmune hemolytic disease are autoagglutination, "warm" autoantibodies, and a marked deficiency of complement.⁶

¹Chauffard, M. D., and Troisier, J.: *Semaine med.* 28: 94, 1908.

²Dameshek, W., Schwartz, S. O., and Gross, S.: *Am. J. M. Sc.* 196: 769, 1938.

³Evans, R. S., Takahashi, K., Duane, P. B., Payne, R., and Lui, C. K.: *Arch. Int. Med.* 87: 48, 1951.

⁴Harrington, W. J., Sprague, C. C., Minnich, V., Moore, C. V., Ahlin, R. C., and Dubach, R.: *Ann. Int. Med.* 38: 433, 1953.

⁵Moeschlin, S., and Wagner, K.: *Acta haemat.* 8: 29, 1952.

⁶Young, L. E., and Miller, G.: *Am. J. M. Sc.* 226: 664, 1953.

Cold agglutinins have been well recognized, especially in cases of atypical (viral) pneumonia,¹ but their presence is generally not associated with abnormal hematologic findings. On rare occasions, hemolytic anemia may accompany such cases,² and Moeschlin and associates³ have reported a pancytopenia in a case of primary atypical pneumonia with incomplete cold hemagglutinins. In some of these cases, it is possible to demonstrate agglutination of red cells in conjunctival vessels by the application of cold to the eyeballs.⁴

Cold hemolysins in paroxysmal cold hemoglobinuria have been known for a long time and account for the Donath-Landsteiner reaction. Other hemolysins have been more recently described, among these the acid hemolysins associated with paroxysmal nocturnal hemoglobinuria.⁵ Other hemolysins may include bacterial, chemical, or infectious agents.

The search for antibodies in autoimmune hemolytic disease continues at an accelerated pace with some investigators still reticent in accepting the idea that the body would produce substances that in effect were committing suicide within certain hematologic arenas. It is perhaps easier to accept those cases in which known modifying agents have played a role but even in these instances the exact nature of the antibody (if "antibodies" they really are) has not been fully established. These substances are globulins or are perhaps found in globulin fractions. In some instances the globulin fraction may be abnormal, but in other instances the abnormal red cell reaction may be due to a normal component. Hinz and co-workers⁶ have described properdin, a normal serum protein, as a specific factor in the destruction of the abnormal erythrocyte in paroxysmal nocturnal hemoglobinuria. However, it requires complement, as well as magnesium ion, for its activity.

In the drug-induced hemolytic anemias, the mechanism which causes the condition has been obscure, but recently Harris⁷ has elaborated the *modus operandi* in a patient with hemolytic anemia due to Fuadin administration for the treatment of schistosomiasis. Three hundred cubic centimeters of the plasma of this patient were injected into a normal recipient. Five days later, the recipient was given Fuadin intravenously, following which gross erythrocyte agglutination occurred, and his red cells became Coombs-positive, with a diminution in titer of circulating agglutinin.

Another method of study of immunologic mechanisms in the hemolytic anemias has been the observation of survival times of the red cells, either by the Ashby technic or using isotope-labeled red cells. In the cases having intracorpusecular defects, injection of these red cells into a normal recipient has revealed shortened survival times, whereas in most instances of extracorpusecular defects, the survival time is normal. On the other hand, the injection of

¹Stats, D., Wassermann, L. R., and Rosenthal, N.: *Am. J. Clin. Path.* **18**: 957, 1948.

²Nelson, M. G., and Marshall, R. J.: *Brit. M. J.* **2**: 314, 1953.

³Moeschlin, S., Siegenthaler, W., Gasser, C., and Hässig, A.: *Blood* **9**: 214, 1954.

⁴Ferriman, D. G., Dacie, J. V., Keele, K. D., and Fullerton, J. M.: *Quart. J. Med.*, N. S. **20**: 275, 1951.

Gördüren, S.: *Brit. J. Ophth.* **30**: 613, 1946.

⁵a. Ham, T. H., and Dingle, J. H.: *J. Clin. Invest.* **18**: 657, 1939.

b. Ham, T. H.: *Arch. Int. Med.* **64**: 1271, 1939.

⁶Hinz, C. F., Jr., Jordan, W. S., Jr., and Pillemer, L.: *Proc. Central Soc. Clin. Invest.* **27**: 57, 1954.

⁷Harris, J. W.: *Proc. Central Soc. Clin. Invest.* **27**: 55, 1954.

normal red cells into a patient with intracorpuseular disease resulted in normal survival, and the injection of normal red cells into a patient with extracorpuseular disease usually resulted in shortened survival time. In many instances of extracorpuseular disease, immunologic processes play a dominant role. Experiments such as these are not within the realm of routine clinical laboratories but are helpful in elaborating the research aspects of these syndromes. In their excellent review of red blood cell survival studies, Eadie and Brown¹ discuss this technic analytically and append a comprehensive bibliography. These authors maintain that random destruction is probably a basic mechanism accounting for the rapid disappearance of red cells in both the intracorpuseular and extracorpuseular diseases.

For elaboration of this field concerning the acquired hemolytic anemias, the reader is referred to articles by the following authors: Dameshek,² Wright and associates,³ and Crosby.⁴ From the clinical standpoint it is well to remember that many of the acquired hemolytic states may be associated with underlying disease, such as collagen disease (including lupus erythematosus), Hodgkin's disease, lymphosarcoma, lymphocytic leukemia, sarcoidosis, reticulum cell sarcoma, and a host of other chronic disease processes, including carcinoma.

Fig. 141 summarizes the approach to study of the hemolytic syndromes in a very graphic manner.⁵

Studies relating to physiopathologic in vivo mechanisms in the production of hemolysis have been reviewed by Wassastjerna.⁶ These include intravascular hemolysis, intravascular agglutination, and erythrophagocytosis, to which might be added erythrocytic fragmentation. In the hemolytic syndromes several of these mechanisms may be operative simultaneously to produce the anemia.

Knowledge of the immunologic aspects of thrombocytopenic purpura in the human being has been of more recent origin than that related to anemia. It has been frequently shown that patients with idiopathic thrombocytopenic purpura have a platelet agglutinin in their plasma which is demonstrable in vitro. Harrington⁷ has summarized the clinical significance of the antibodies for platelets, pointing out that (1) these agglutinins play a major role in the production of the thrombocytopenic state; (2) platelet agglutinins may appear after repeated transfusions; and (3) platelet agglutinins may appear as a sequela of pregnancy. The initial phase of Harrington's research in this field is a dramatic story which has opened the door to a brilliant research problem. To prove that the plasmas of patients with idiopathic thrombocytopenic purpura have a plasma factor which will induce thrombocytopenia, he had injected into himself such plasma, and promptly experienced the clinical and hematologic picture of thrombocytopenic purpura.

¹Eadie, G. S., and Brown, I. W.: *Blood* 8: 1110, 1953.

²Dameshek, W.: *Proc. Internat. Soc. Hemat.*, New York, 1950, Grune & Stratton, p. 120.

³Wright, C. S., Sundhariglati, B., Bass, J. A., and Bunner, A. E.: *Arch. Int. Med.* 92: 357, 1953.

⁴Crosby, W. H.: *Bull. New York Acad. Med.* 30: 27, 1954.

⁵Wright, C. S.: *Mod. Med.* 22: 73, 1954.

⁶Wassastjerna, C.: *Blood* 8: 1042, 1953.

⁷Harrington, W. J.: *Sang* 25: 712, 1954.

From this beginning, the experiments have been elaborated both in vivo and in vitro. In 72 patients with idiopathic thrombocytopenic purpura, he found 50 who showed positive agglutinins and 22 who were negative for agglutinins. In the acute cases, 30 out of 43 were positive, and in the chronic variety 20 out of 29 were positive for agglutinins. Autoagglutinins were not

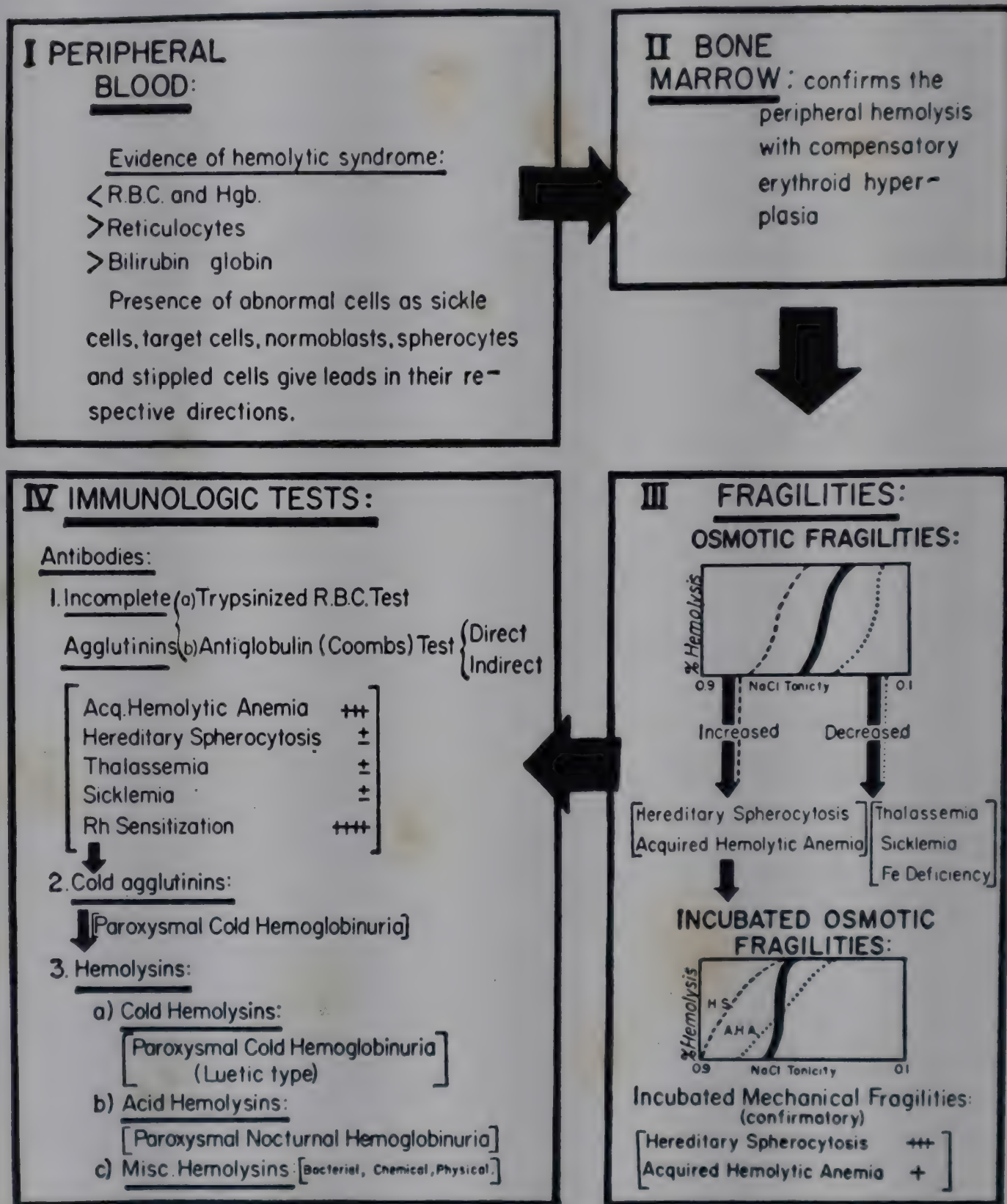


Fig. 141.—The hemolytic syndromes. Laboratory differentiation of hemolytic syndromes. Peripheral blood (I) and the marrow (II) indicate a hemolytic process. Osmotic fragilities (III) differentiate hereditary spherocytosis and acquired hemolytic anemia, on the one hand, from thalassemia, sickleemia, and iron-deficiency anemias on the other. Hereditary spherocytosis (H.S.) and acquired hemolytic anemia (A.H.A.) are differentiated from each other by the incubated osmotic and mechanical fragility tests. The cells from hereditary spherocytosis show a pronounced increase in fragility (broken line). The cells from acquired hemolytic anemia show less change and usually assume an asymmetric curve (dotted line) which may cross over the normal incubated control. If the diagnosis is still not evident, immunologic tests (IV) are done. (From Claude-Starr Wright: *Mod. Med.* 22: 78, 1954. By permission of the author and the publisher.)

tested in all of these cases because in some instances only serum was available, but where both platelets and serum of the patients were available, two-thirds of the cases (27) showed autoantibodies. In addition to idiopathic thrombocytopenic purpura, autoantibodies were demonstrated in 2 cases of chronic lymphocytic leukemia, 2 cases of lupus erythematosus disseminata, a case of carcinomatosis, a case of atypical acquired hemolytic anemia, and a case of drug sensitivity. These cases represent 7 out of 13 controls with secondary thrombocytopenia in whom it was possible to test for autoantibodies. In this group of secondary thrombocytopenias, 28 out of 57 patients tested showed positive agglutinins in their sera. However, these agglutinins included both iso- and autoagglutinins. Of importance here is that only 3 out of the 28 positives had never been transfused or been pregnant. The significance of previous pregnancy or transfusions will be discussed presently. In 144 cases of nonthrombocytopenic controls, only 16 displayed agglutinins of any kind, but only 2 of these 16 had never been transfused or pregnant. In 75 patients of the nonthrombopenic control group, there was not a single instance in which autoagglutinins were found. The patients within these control groups with positive agglutinins are considered chiefly as examples of isoagglutination, occurring mainly as the result of multiple transfusions.

Harrington and his co-workers¹ have reported eight types of platelets; others² recognize four groups of serologically distinct platelet groups. These various platelet types have antigenicity in the same way as red cell types and transfusions of "mismatched platelets" will result in antibody formation. Apparently, isoagglutinins for platelets can also be induced during pregnancy in a manner similar to that responsible for Rh incompatibility between the mother and the fetus.

The finding of autoantibodies in the blood of patients with thrombocytopenic purpura poses a number of questions. Clinicians have constantly been in search of criteria for splenectomy in these patients, and serologic tests of the type being discussed here were immediately looked upon as possible aids in this direction. Thirty-eight out of the 50 patients with positive agglutinins were splenectomized. Twenty-five of these had a complete remission, 4 patients had a partial remission, and the remaining 9 were not benefited by surgery. In the group of 22 patients without demonstrable agglutinins, 16 were splenectomized with the following results: 2 complete remission, 4 partial remission, 10 not benefited. Although these figures indicate that the prognosis with splenectomy is apparently better when agglutinins are demonstrable, Harrington cautions that "more experience is needed, however, before it would be permissible to state with certainty that the presence or absence of platelet agglutinins should be a major criterion for the selection of candidates for splenectomy."

Autoantibodies in a mother may result in neonatal thrombocytopenia in infants; Harrington cites 9 such cases. This may occur even though a remission has been induced in the mother by splenectomy. Harrington questions the utility of platelet transfusions in patients showing autoantibodies since

¹Harrington, W., et al.: *Ann. Int. Med.* 38: 433, 1953.

²Stefanini, M., Plitman, G., Dameshek, W., Chatterjea, J. B., and Mednicoff, I. B.: *J. Lab. & Clin. Med.* 42: 723, 1953.

platelets disappear readily from the circulation of such patients. He would reserve such measures only as temporizing in patients presenting serious hemorrhage intracranially or within the gastrointestinal tract. Harrington warns against the use of patients with a history of thrombocytopenic purpura as blood donors since antibodies may persist for years even though remission has been induced by splenectomy.

Isoimmunization may create problems akin to autoimmunization. Since multiple transfusions may induce isoimmunization, individuals receiving such transfusions should be screened as future donors of whole blood or plasma. Isoimmunization may occur in pregnancy as previously noted. Harrington suggests that "rejection of all candidates (for blood donors) who have received more than 20 transfusions of whole blood or anyone who has been transfused or pregnant within one year would seem to be reasonable minimum precautions at the present time." Harrington points out the lack of feasibility of protracted platelet transfusions in patients with thrombocytopenic purpura, especially of the secondary type; these patients develop isoimmunization and even refractoriness after three to six such transfusions and further pursuit of such therapy is valueless.

A third problem related to isoimmunization is primary neonatal isoimmunization, occurring in infants born to mothers who have not had thrombocytopenic purpura. Isoimmunization in the mother may have come about through previous transfusions, pregnancy, or both. Such isoimmunizations correspond with that seen with Rh incompatibility in that the mother displays no symptoms.

As technics improve, agglutinin detection in the thrombopenic states will improve. Adelson and Stefanini¹ describe a heterologous (Forssman) type of platelet agglutinin in the case of thrombotic thrombocytopenic purpura. Phase contrast microscopy studies² of the bone marrow of normal recipients of plasma from a patient with a high-titer platelet agglutinin revealed changes in the megakaryocytes of the recipients similar to those seen in idiopathic thrombocytopenic purpura. These observations suggest that the antiplatelet factor acts by (a) attacking platelets being discharged from megakaryocytes, (b) preventing normal platelet formation from megakaryocytes, and (c) causing maturation arrest of megakaryocytes.

The immunologic association of leukopenia and antibody formation directed at leukocytes is relatively small as compared with the large body of evidence related to the red cells and platelets. The field is particularly well covered from the standpoint of bibliography, technic, clinical material, and experimental studies by the excellent paper of Dausset, Nenna, and Bracy.³ These authors present 19 cases of leukopenia in which leukoagglutinins were demonstrable out of 2,000 normal and 500 patients' sera. In 18 out of these 19 cases, neutropenic leukopenia was present. In general, the titer of agglutinin more or less reflected the degree of leukopenia. In a case of Pyramidon sensitization, the leukoagglutinin disappeared along with the neutropenia. Transfusion of blood containing leukoagglutinins resulted in leukopenia in

¹Adelson, E., and Stefanini, M.: *Blood* 7: 700, 1952.

²Pisciotta, A. V., Stefanini, M., and Dameshek, W.: *Blood* 8: 703, 1953.

³Dausset, J., Nenna, A., and Bracy, H.: *Blood* 9: 696, 1954.

the donors. A rather unusual feature of this study is that in most of these patients, leukopenia, anemia, and thrombocytopenia were all present, but in only 4 cases were red cell antibodies found and in only 1 case was a platelet agglutinin demonstrable. These observers offer several possible answers to this dilemma, emphasizing our incomplete knowledge in this field. A rather striking feature of these cases is that the bone marrow is usually hypoplastic with maturation arrest at various levels of myelocytic development. This is in sharp contrast with the observations of hyperplasia seen in the hemolytic anemias and idiopathic thrombocytopenic purpuras. These authors draw parallelism between the hemolytic anemias and the leukopenias, with those cases in which no infection, intoxication, or other pathologic processes were present,

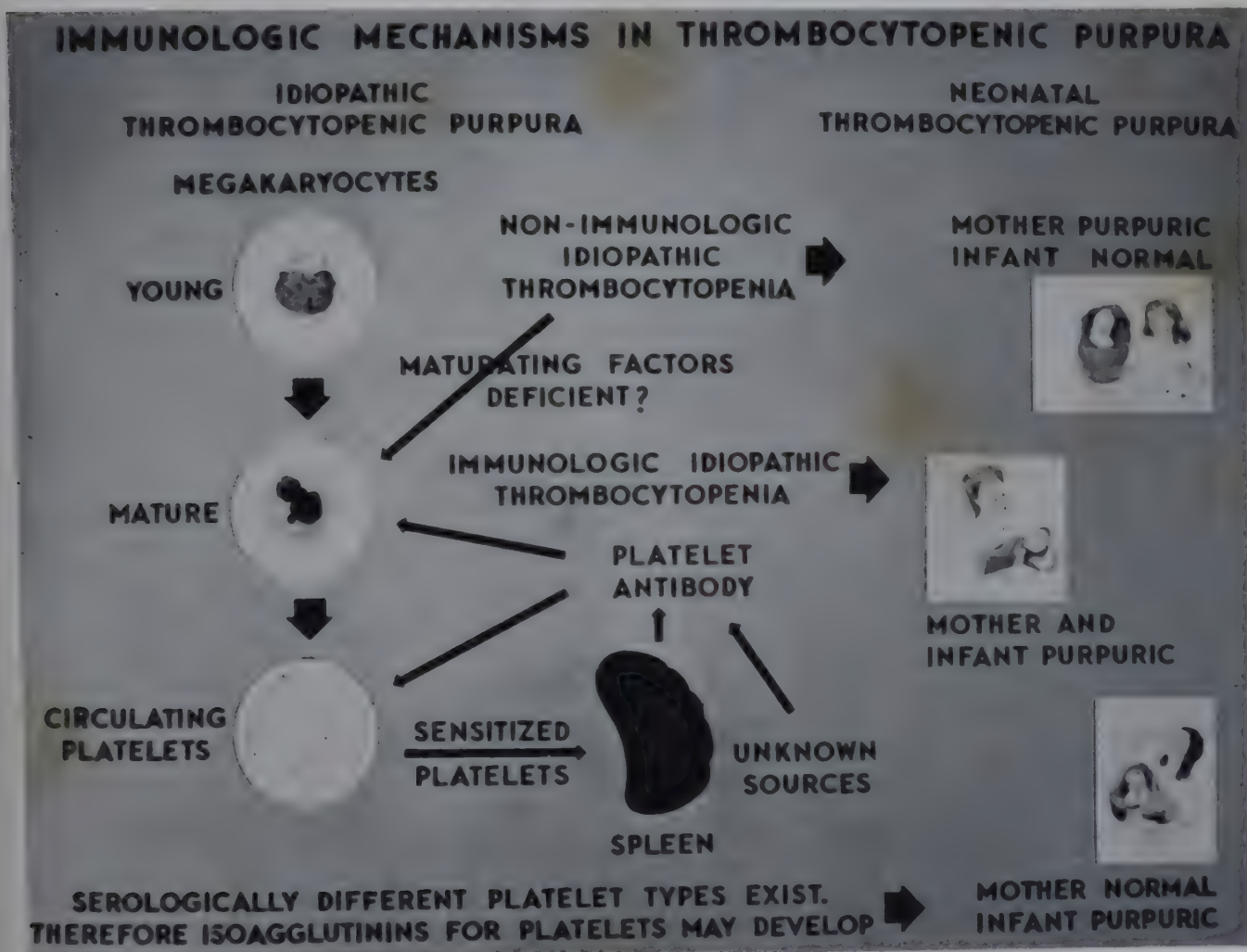


Fig. 142.—Pictorial presentation of current concepts of the pathogenesis of idiopathic and neonatal thrombocytopenic purpura. (From Harrington, W. J.: *Sang* 25: 712, 1954. By permission of the author and the publisher.)

corresponding to the idiopathic acquired hemolytic anemias with autoantibodies. They suggest designating this group "chronic idiopathic pancytopenia with leukoagglutinin." Cases associated with acute leukemia, Hodgkin's disease, and lymphosarcoma would correspond to the secondary acquired hemolytic anemias and could be called "symptomatic pancytopenias with leukoagglutinin." The exhaustive character of this study points up many of the unsolved problems in the field of immunohematology and we heartily recommend reading it.

Attention has been previously called to immunopancytopenia in a patient with atypical pneumonia.¹ In this case, leukoagglutinins were not demon-

¹Moeschlin, J., et al.: *Blood*, 9: 214, 1954.

strable in vitro. However, transfusion of 300 c.c. of blood from this patient resulted in a prompt fall in leukocytes of the donor. In previous experiments, Moeschlin and Wagner² demonstrated in vitro agglutination of leukocytes by the serum of a patient with amidopyrine-induced leukopenia. If blood from this patient was injected into a normal recipient, no effect on the leukocyte level was noted. However, if the same recipient was "primed" by administering 5 grains of amidopyrine 3 hours prior to the administration of the blood, a profound neutropenia followed. This experiment corresponds to the findings in thrombocytopenic purpura³ with quinidine sensitization.

NORMAL COMPOSITION OF THE BLOOD

The circulating blood normally consists of a suspension of blood cells of different kinds in a fluid element called "plasma." The formed elements are erythrocytes, leukocytes, and blood platelets. Plasma is the liquid portion of blood, of a very complex composition: water, proteins, carbohydrates, lipids, electrolytes, other mineral salts, hormones, antibodies, and enzymes. If blood is allowed to clot, fibrinogen is removed from the plasma, and the remaining fluid is called "blood serum."

The total volume of the blood comprises about 6 to 8 per cent of the body weight in adults.

There are normally from 4,600,000 to 6,200,000 red blood cells per cubic millimeter in the adult male (average 5,400,000) and from 4,200,000 to 5,400,000 in the adult female (average 4,800,000). There are from 5,000 to 10,000 leukocytes normally, with an average of 7,000 to 8,000 per cubic millimeter. The number of blood platelets varies from 200,000 to 300,000 per cubic millimeter of blood according to some authorities, and is higher in men than in women and children.

Red Blood Cells, or Erythrocytes

The normal red blood cell or erythrocyte is a circular to slightly oval, biconcave disc with an average diameter of 7.2 μ . It is orthochromatic. It is bright yellow with a central depression, highly light-refractile and apparently homogeneous. The red cell (cellular element) in the circulating blood is normally nonnucleated. Red cells originate in the bone marrow substance. Jordan and Baker held that the red cells are released into the circulation through gaps in the endothelial walls of the capillaries. The parent cell of the erythrocytes in the bone marrow is nucleated. This parent cell, according to Schilling, is an erythrogonia; according to other authorities, it is an erythroblast. In its development, the nucleus becomes smaller, and the chromatin more compact. The cell then becomes a normoblast or bone marrow nucleated cell. It loses its nucleus either through extrusion from the cell, or by dissolution. Just when it loses its nucleus before entering the circulation is not definitely known.

²Moeschlin, S., and Wagner, K.: *Acta haemat.* 8: 29, 1952.

³Steinkamp, R. S., Moore, C. V., and Doubek, W.: *J. Lab. & Clin. Med.* 45: 18, 1955.

The following are some of the attributes of red blood cells¹:

	<i>Average</i>	<i>Range</i>
Diameter:	7.5 μ	7.2 to 7.8 μ
Thickness:	2.0 μ	1.7 to 2.2 μ
Volume:	87.0 c. μ	70 to 94 c. μ
Hemoglobin content:	29 micromicrograms	23 to 35 micromicrograms

There is a difference of opinion among hematologists as to the origin of the red cells. Schilling's view is that the red cell comes from an "erythrogenia," then a megaloblast, then a normoblast or mature nucleated or bone marrow cell, then the nonnucleated polychromatic erythrocyte, and finally the mature red cell found in the circulation, the erythrocyte. Downey, Piney, and Kato believe that there are two separate series of red cell progenitors. They call one the megaloblast series, originating in a so-called promegaloblast, and believe that it develops through a megaloblastic stage to a large nonnucleated cell which they term a megalocyte. The other series they term a normoblast series, believing that it begins with a cell which they call a pronormoblast or a normoblast, and develops through the stage of a normoblast into the erythrocyte of normal blood. They contend that the megaloblast series develops early in fetal life as a method of red cell formation and does not appear after birth in any condition other than pernicious anemia, but that in pernicious anemia the formation of red cells reverts to that early fetal form. In this connection, Osgood² refers to the views of Downey and others concerning the erythrocytic cells in pernicious anemia which show a very immature nuclear structure with much hemoglobin in the cytoplasm. Since, in the process of maturation, the basophilia of the cytoplasm decreases as the hemoglobin formation increases, these authors regarded these cells in pernicious anemia as more mature than those with little hemoglobin in the cytoplasm. Osgood does not believe that the amount of hemoglobin in the cytoplasm of red cells justifies any classification as to age, since nonnucleated erythrocytes sometimes contain practically no hemoglobin, and these are assuredly more mature than nucleated red cells containing much hemoglobin. Osgood suggests the use of the nucleus alone as the criterion of the maturity of the cell, thus giving a continuous series of cells with perceptible ageing, from the megaloblast (karyoblast) through the mature normoblast (metakaryocyte) which is just losing its nucleus. Osgood has found in normal marrows and in marrows of many diseases other than pernicious anemia megaloblasts identical in all respects to those seen in the marrow of pernicious anemia, although they are much less numerous, requiring the examination of thousands of cells. Furthermore, if antipernicious anemia principle is administered to patients with pernicious anemia, a gradual transformation results, which can be followed by daily sternal marrow examinations, or by examining hourly cultures of pernicious anemia marrow. Here one sees transformation of typical immature megaloblasts, or promegaloblasts of Downey, into typical normoblasts. It does not seem logical that deficiencies occurring in mature life produce reversions to embryonic type of blood formation, or that the administration of the deficient substance causes return to adult type of erythrocytic formation. Osgood, therefore, believes with Isaacs and Sabin that the megaloblast

¹Albritton, E. E.: *Standard Values in Blood*, Philadelphia, 1952, W. B. Saunders Co.

²Osgood: *Atlas of Hematology*, San Francisco, J. W. Stacy, Inc., p. 13.

blast is the precursor of the normoblast, with the actual progression down through the megaloblast series, then through the normoblast series, rather than having these two series parallel each other.

Hemokonia

In the dark-field preparation, we find *hemokonias*, which are fine, dust-like particles with active ameboid movement. Neumann¹ considers these bodies fatty particles and notes that they are prevalent particularly after eating. (See page 13.)

Hemoglobin

Hemoglobin is the red, iron-bearing protein contained within the erythrocytes in normal blood. Its major function in the normal individual is as a carrier of oxygen from the lungs to the tissues, where it readily releases this oxygen to the tissues and then returns to the lungs to combine with more oxygen. This respiratory function of hemoglobin is vital to the life of all cells. Although not all of the physicochemical aspects of hemoglobin have been established, certain basic facts are generally accepted: Hemoglobin consists of four heme molecules conjugated with one globin molecule. The globin fraction is a complex protein with a molecular weight between 63,000 and 67,000. The source of globin is protein in the diet, which is broken down in the gastrointestinal tract to amino acids of various types, which in turn are absorbed and used in hemoglobin synthesis. Although some of the amino acids found in globin have been identified as arginine, histidine, lysine, tryptophane, and tyrosine, it is believed that no one single amino acid is alone essential for hemoglobin synthesis. Orten and Orten² have demonstrated that if any one of the essential amino acids is added to the diet, there is no increase in hemoglobin synthesis. The amount of dietary protein required to keep the body in satisfactory equilibrium so far as hemoglobin synthesis is concerned is approximately 1 gram of protein per kilogram of body weight. Whipple,³ in his classical experiments, demonstrated that if protein is not obtained from outside the body in the diet, then the body will selectively take protein from tissue in order to synthesize hemoglobin: "the body guards jealously the fabrication of the hemoglobin, and given a need for both plasma protein and hemoglobin, the protein flow favors hemoglobin synthesis." Practical use of this was made in the care of the wounded under the program known as "Reparative Surgery" during World War II.⁴

Not only will inadequate food intake affect the amount of hemoglobin formed, but poor absorption of this protein from the gastrointestinal tract will also affect the amount of hemoglobin absorbed; therefore, such gastrointestinal affections as protracted diarrhea in colitis, atrophic gastritis, and other conditions will result in hemoglobin deficiency. There is present in the gastrointestinal tract intrinsic factor and erythrocyte maturation factor which

¹Neumann: Wien. klin. Wchnschr., p. 28, 1907.

²Orten, A. U., and Orten, J. N.: Nutrition 30: 137, 1945.

³Whipple, G. H.: Am. J. M. Sc. 203: 477, 1942.

⁴a. Churchill, E. D.: Ann. Surg. 120: 266, 1944.

b. Hampton, O. P.: Ann. Surg. 122: 289, 1945.

c. Hampton, O. P., Jr.: Ibid. 123: 1, 1946.

d. Hampton, O. P., Jr.: Ann. Surg. 123: 238, 1946.

e. Lyons, C.: J. A. M. A. 123: 1007, 1943.

affect hemoglobin formation. In some unknown way these factors are related to the fabrication of hemoglobin through the intermediary role of the liver prior to the taking up of the substances in the bone marrow by the red blood cells.

The four heme molecules of hemoglobin consist of four protoporphyrin molecules containing ferrous iron. While iron is important in the synthesis of hemoglobin, exceedingly small amounts are required in the diet for normal hemoglobin synthesis.

Hemoglobin consists of four heme molecules, chemically identified as ferrous protoporphyrins. The chemical alignment of these porphyrins in relation to globin in the formation of hemoglobin has been extensively studied by Pauling.⁵ The iron required for this hemoglobin synthesis from the *diet alone* is almost infinitesimal, being perhaps approximately 12 mg. as the daily average minimum requirement. This amount is readily available from any average diet; it requires an unusually subnormal diet to bring iron intake to levels lower than this. The reason for this is that the iron stored within the body is not depleted unless there is loss through recurrent hemorrhage or a great increase in demand such as occurs in early childhood, during the growing period, and in pregnancy. When extra iron is needed, it may be given in the form of iron salts which are absorbed from the gastrointestinal tract in the ferrous state. Under unusual circumstances, parenteral iron therapy may be necessary to supply the depleted iron stores of the body.

Iron combines in the small intestine with a protein, forming "ferritin." It is then released from the mucosal cells of the small intestine to the plasma, where it has been shown that beta one globulin carries the iron in combination known often as "transferrin." Associated with this globulin, it is transported to the bone marrow where the iron combines with protoporphyrin to form heme. The heme then combines with globin to form hemoglobin.

Hemoglobin normally is in the state in which it has absorbed oxygen, and is known as **oxyhemoglobin**. Oxyhemoglobin can be identified in the spectroscopic by the two intense absorption bands at 578 and 540 m μ . All hemoglobin compounds absorb light intensely at the shorter wave lengths and weakly at the longer wave lengths. This disproportion in absorbence accounts for the red color of hemoglobin solutions. Spectroscopic analysis may be used for the identification of some of the abnormal hemoglobin derivatives.

Abnormal Forms of Hemoglobin.—

Methemoglobin is an abnormal form of hemoglobin. It is usually associated with intoxication with chlorates, nitrites, nitrobenzol, aniline, hydrogen sulphide, and others. It may be formed from exposure in industry to excess amounts of these agents, or may result from excessive intake of drugs, or in idiosyncrasies to certain drugs, such as acetanilid and sulfanilamide. It is formed by some bacteria. When methemoglobinemia is present, the patient becomes cyanotic and the blood is usually chocolate colored. Methemoglobin does not have the same capacity to take up oxygen as the normal hemoglobin, and thus the respiratory processes are impaired. Spectroscopic examination

⁵Pauling, L.: Hemoglobin, Stanford M. Bull. 6: 215, 1948.

of the blood will show the identifying band at $630\text{ m}\mu$ for methemoglobin. It has four absorption bands, 630, 578, 540, and $500\text{ m}\mu$. Sunderman and co-workers⁶ suggest that if this band is present, upon addition of 5 mg. of KCN or sodium sulphite, the band will disappear. In examination of blood for methemoglobin, it must be remembered that methemoglobin is present in both the plasma and the red cells, and these should be examined separately. It can also be found in the urine.

Carboxyhemoglobin (carbon monoxide hemoglobin) results from a combination of hemoglobin and carbon monoxide. It has been claimed that hemoglobin has 200 times the affinity for carbon monoxide that it has for oxygen. Carboxyhemoglobin is found in the blood after exposure to inhalation of artificial gas, automobile exhausts, underground gas collections, or other agents containing carbon monoxide. It is believed that life can be maintained with a 20 per cent concentration of carbon monoxide hemoglobin, but that a concentration of 50 per cent or greater will result in death.

Carboxyhemoglobin may be identified by spectroscopic examination. It must be remembered that the bands are at 572 and $535\text{ m}\mu$, which are very close to those of oxyhemoglobin. Sunderman has suggested the following spectroscopic test:

Dilute the blood with water until the two bands in the spectrum of both carbon monoxide hemoglobin and oxyhemoglobin are still clear but faintly visible.

Add 5 mg. of dithionite ($\text{Na}_2\text{S}_2\text{O}_4$).

Mix gently and examine for persistence of the bands of carboxyhemoglobin. If two bands persist, carboxyhemoglobin is present.

Other tests are given in the chapter on Toxicology. See also page 1161.

Sulfhemoglobin is an abnormal hemoglobin, the exact chemical structure of which is unknown. Sulfhemoglobinemia often accompanies methemoglobinemia. It has been described in patients addicted to taking Bromo-Seltzer, a common headache remedy, and in others who have either been exposed to large doses of phenacetin and the sulfonamides, or who have idiosyncrasies to these drugs.

Sulfhemoglobin shows spectroscopic absorption bands at 618, 578, and $540\text{ m}\mu$. It is found in both the red blood cells and the plasma. Unlike methemoglobin, addition of 5 per cent cyanide (KCN) will not cause the band to disappear. Sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) also will leave the band unchanged. Sodium sulphite and carbon monoxide will leave the band unchanged, but sodium sulphite with sodium hydroxide added causes disappearance of the band as does 3 per cent hydrogen peroxide.

In the catabolism of hemoglobin, the major portion of the pigment is broken down ultimately to form bile pigments. However, small amounts of porphyrin, coproporphyrin, and uroporphyrin are excreted in the urine and the stool. When excessive amounts of porphyrin are excreted in the urine, the condition is referred to as "**porphyrinuria.**" Excessive amounts of these hemoglobin derivatives have been described in a great variety of pathologic

⁶Sunderman, et al.: J. Clin. Path. 23: 1953.

conditions such as lead poisoning, pellagra, severe infections, liver disturbances, chronic alcoholism, leukemia, Hodgkin's disease, pernicious anemia, hemolytic jaundice, and aplastic anemia.

The **porphyrins** have been the subject of extensive studies by Watson and Larson.⁷ In addition to the occurrence of porphyrinuria in these patients, excessive amounts of porphyrins may occur in porphyria. Porphyria is a disturbance of pyrrol metabolism and may be either congenital or it may occur in an acute form. The high incidence of porphyria in the Scandinavian countries has made this a subject of extensive studies by Waldenström and Vahlquist.⁸

Congenital porphyria is a congenital defect of metabolism with great sensitivity to light and accompanied by the excretion of large amount of type I porphyrins. These patients show bullous lesions of the skin following exposure to light. The slightest irritation of the skin is followed by marked redness and edema of the exposed surface. Round vesicles appear over the affected area, accompanied by pustules and necrosis. The condition may be so severe that loss of the fingers, nose, or ears may occur. The teeth appear bright red and sometimes brown or yellowish. By ultraviolet light measurement, considerable deposit of porphyrin has been seen in the dentine.

The urine excreted in these cases is Burgundy red with a brown tinge, but sometimes varies from pale pink to almost black. It may be normal yellow when passed but darkens on exposure to light. When the feces contain large amounts of porphyrin, they turn almost black on oxidation.

Acute porphyria is apparently congenital. Patients with this condition show no light sensitivity and porphobilinogen is excreted in the urine in large amounts. The disease is inherited as a Mendelian dominant and is more common in women than in men. They show the following symptoms: abdominal symptoms, with severe, cramplike pains, vomiting, and pronounced constipation. The abdomen is distended and there may be slight fever and tachycardia. The skin may show a diffuse or spotted pigmentation. The nervous system shows involvement by reason of constant pain in the extremities, accompanied by muscular weakness. There is paralysis in the lower motor neuron type with flaccidity and loss of tendon reflexes. The symptoms suggest Landry's paralysis. Mental symptoms may suggest hysteria. Some patients show toxic delirium with hallucinations of vision and hearing. The urine is dark but may contain only the colorless porphyrinogen.

The tests for porphyrins are given on page 79. See also page 1161.

The normal urine contains coproporphyrin type I and coproporphyrin type III, which are present only in traces and do not color the urine. Coproporphyrins types I and III are not derived from the protoporphyrin of hemoglobin but are members of the porphyrin groups of the body. In acute porphyria, which is a rare congenital disease due to an abnormality of purine metabolism, the urine may be a deep red color with variable amounts of coproporphyrin.

The true abnormal compounds found in the urine are uroporphyrins and porphobilinogen. According to Waldenström, porphobilinogen is neither a

⁷Watson, C. J., and Larson, E. A.: *Physiol. Rev.* 27: 478, 1947.

⁸Waldenström, J., and Vahlquist, B.: *Acta med. scandinav.* 117: 1, 1944.

pink, red-brown, or black, depending on the concentration. It reacts with Ehrlich aldehyde reagent, giving a deep red color in chloroform, but soluble in water. Porphobilin does not give this reagent and is not fluorescent.

TABLE 47.—DISEASES FROM PIGMENT DISTURBANCES

TERM TO BE USED	TERMINOLOGY
Blood pigment disturbance in plasma, urine due to	
Unknown cause and type	
Hemoglobinuria, due to	
Unknown cause	
Paroxysmal hemoglobinuria, due to	
Unknown cause	Marchiafava-Micheli
Nocturnal	"e frigore"
Cold	March hemoglobinuria
Exertional	
Allergic	
Favism	
Myoglobinuria, due to	Paralytic myoglobinuria
Unknown cause	
Crush syndrome	
Poison	Haff disease
Methemoglobinemia, due to	
Unknown cause	
Poison	
Aniline derivative	
Acetanilid	
Heredity	
Sulfhemoglobinemia, due to	
Unknown cause	
Poison	
Carbon monoxide hemoglobinemia	
Constitutional hyperbilirubinemia	
Nonhemoglobin pigment disturbance in plasma, urine, due to	
Unknown cause and type	
Porphyria, generally	
Porphyria, due to	
Unknown cause	
Photosensitive porphyria	
Acute toxic (intermittent) porphyria	
Porphyria due to drugs (specify)	
Carotinemia	

Reproduced from the Am. J. Clin. Path. 20: 574-579, 1950, of the Williams and Wilkins Company, Baltimore.

Acute porphyria is also attended by excretion of u

eds of Great Britain during World War II. Sonder-
mend examining urine in such cases for absorption.
Since these bands are very close to those of oxyhemo-
make a positive diagnosis by the spectroscope alone.
e to 10 c.c. of urine and place it in a 9 (Gradacol Elford)

, a clear filtrate is obtained, whereas in the presence of myo-
with an absorption band at $583\text{ m}\mu$ is obtained.

Leukocytes

various sizes and shapes, as will be discussed later.
various hematopoietic organs:

system, consisting of the marrow of the short bones
conditions, also the bone marrow of the long tubular
ullary centers (myelogenous transformation of spleen
ocytic system forms granulocytes (certain forms of

ytic system, consisting of spleen, glands, and dispersed
mphocytic system forms the lymphocytes.

helial system, consisting of reticuloendothelial tissue
one marrow, and connective tissue (Aschoff-Kiyono's
oendothelial system furnishes the large mononuclears
under the Schilling classification, the "monocytes."
eukocyte count of the adult is 7,400 per cubic milli-
5,500 to 11,000 per cubic millimeter. Variations occur
ccess beyond 11,000 being called "leukocytosis," and
'leukopenia.'" Variations may occur in the blood of
n in health, from day to day or at different hours of
uthorities have spoken of "digestive leukocytosis,"
ith food intake. There is some question as to whether
such a designation since variations may occur quite

ed the differences between leukocyte counts in indi-
e exercise and found that there is no such thing as an
ocyte level and that fluctuations in the leukocyte pic-

hour period in bed appears to bring about a more irregular distribution of the leukocytes, both in the total and in the differential count, than is present during a state of normal activity. The greatest care should be exercised whenever an attempt is made to interpret the significance of the leukocytic picture when the fluctuations noted are within a normal range.

Medlar's observations emphasized that while it is useful to know whether the leukocyte count is elevated or diminished, these facts alone are insignificant in comparison with the value of the knowledge of the entire blood picture. The differential leukocytic picture should always be considered in conjunction with the total leukocyte count. Schilling and Gradwohl have emphasized that if it is impossible to have both the total leukocyte count and the differential count, the latter should be greatly preferred over the former.

There are instances in which there may be prevalent a physiologic leukocytosis. At birth, the leukocyte count averages 18,100 per cubic millimeter, with a range of 9,000 to 30,000 per cubic millimeter. At the end of one week, there is a drop in the total leukocyte count to an average of 12,200, with a range of 5,000 to 21,000 per cubic millimeter. These levels tend to persist until the end of the first or second year and then continue to fall within the normal average. Until the age of 6 to 8 years has been reached, there is a tendency toward the slightly higher leukocyte counts within the normal adult range.

The white count does not seem to be affected appreciably by altitude. During pregnancy, there is usually an elevated count of approximately 10,100 to 10,600, or even as high as 15,500 per cubic millimeter. By the fifth to ninth week post partum, there is a return of the total leukocyte count to within the normal average and normal range.

Blood Platelets

Blood platelets appear in the stained specimen as single, bright, azure, rounded or elongated small bodies with a delicately striped and granular structure. They are about one-half to one-fifth the size of red cells. There is considerable dispute as to their histologic position. Some claim that they are fragments of leukocytes. Others claim that they are broken-off pieces of the pseudopodia of the cytoplasm of megakaryocytes, or giant bone marrow cells (Wright's view). Hayem believes they are primary forms of red cells. Schilling contends that they are parts of the nuclei of the bone marrow red blood cells which have been extruded when the red blood cells have reached maturity.

They were first described by Max Schultz¹ as definite bodies, although Zimmerman² studied them by means of magnesium sulphate used as an anti-coagulant. Hayem in 1878 considered them primary forms of red cells, calling them "hematoblasts." Bizzozero³ gave them the name "blood platelets" and emphasized the connection between these bodies and blood coagulation.

¹Schultz, Max: Arch. f. mikr. Anat. I, 1, 1930.

²Zimmerman, G.: Rust's Mag. f. d. ges. Heilk. 66: 171, 1846.

³Bizzozero, J.: Virchows Arch. 1882, Sc. 261.

There is considerable dispute as to the source of blood platelets. Some have claimed that they are derived from leukocytes but this theory has been disproved by the fact that specific antiserum from blood platelets has no effect on leukocytes or red blood cells. A theory which numbers Schilling as its leading exponent is that they represent extruded nuclei of red cells. The preponderance of opinion seems to favor the correctness of the theory of Wright,⁴ namely, that they are remnants of broken-off pseudopodia of megakaryocytes. Wright noted that blood platelets are found only in animals which have megakaryocytes and that the platelets do not appear in embryonic blood until the megakaryocytes occur. Naegeli made an exhaustive study of the embryology of blood platelets. He stated that they appear in the embryo simultaneously with the megakaryocytes and they both are found in all mammals but not in vertebrates of other species. Schilling⁵ contends that they appear simultaneously with the denucleation of the erythrocytes. Doan and Sabin found that in experimentally induced tuberculosis of the marrow in rabbits, destruction of megakaryocytes was accompanied by a reduction in the number of platelets in the blood. They found, too, that with regeneration of the megakaryocytes the platelets increased in numbers.

To turn to those who believe that the Wright theory is incorrect, let us note that Pianese found a great increase in the number of blood platelets in blood shut off from the circulation in doubly-ligated veins. Brown believes that platelets arise from endothelial and mononuclear cells in the marrow, spleen, and blood. Bunting thinks they are derived from leukocytes and Watson has revived the theory that they represent the altered stroma of degenerating erythrocytes. His work as set forth by Magner⁶ indicated "(1) that rabbits' erythrocytes which have been damaged by intravenous injections of phenylhydrazine, and the erythrocytes of some commonly normal animals, when suspended in platelet counting fluid, undergo a process of degeneration with production of blue-staining granular bodies which are indistinguishable from blood platelets; (2) injection of suitable doses of phenylhydrazine into the circulation of rabbits causes an abrupt rise in the platelet count with a simultaneous fall in the erythrocyte count. The increase in platelets is progressive while the number of erythrocytes is falling. The platelet count begins to recede when the original crop of erythrocytes which were damaged by the injections has disappeared from the circulation. The platelet count may fall to a subnormal figure when the injured cells have all been replaced by young cells."

It is interesting to note that Rosenthal and Falkenheim found that an erythrocytic immune serum is highly agglutinative for red cells but has little effect on young cells and they concluded that platelets and leukocytes have a common origin.

The principal function that is definitely known concerning platelets is their role in blood coagulation. It has been definitely proved that for the production of thrombin, certain substances must be liberated from disintegrated blood platelets. It has been shown, too, that oxalated plasma which

⁴Wright, J. H.: Boston M. & S. J. 154: 643, 1906.

⁵Schilling, V.: Handbuch der normalen und pathologischen Physiologie, Berlin, 1931, Springer.

⁶Magner, Wm.: Textbook of Hematology, Philadelphia, 1938, P. Blakiston, Inc., p. 11.

has been filtered through a Berkefeld filter, and therefore no longer possesses the capacity to coagulate, will undergo coagulation with the addition of blood platelets. Increase in blood platelets is associated with increased coagulability of the plasma when plasma is recalcified. There is also a function of blood platelets connected with blood clot retraction. Rosenthal⁷ thought in some cases of purpura that clot retraction was dependent upon the presence of normal platelets and that even with a larger number of blood platelets present clot retraction may not occur. He believed that this is due to the actual weakness of the platelets, and that the process of clot retraction is hastened by blood platelets.

The question of whether or not blood platelets are increased following operative procedures is discussed on page 561.

Summarizing, most American hematologists believe in the megakaryocytic origin of blood platelets. Schilling⁸ claims that they represent the remnants of extruded nuclei of red cells. Some believe that blood platelets are tissue elements—a product of differentiating cellular activity, perhaps a broken-off fragment of a megakaryocyte or an osteoblast, or even a Kupffer cell, or lymphatic cell fragment. They have also been called Ferrata cells or primary bone marrow cells. Cesaris-Demel contends that when the promegakaryocyte comes in contact with blood plasma, platelets are formed and the giant bone marrow cell therefore attains full maturity. The opponents of the Wright theory⁹ offer the argument against it that we seldom find a blood platelet or giant blood platelet in normal bone marrow.

SUMMARY OF THE CHANGES IN BLOOD CELLULAR ELEMENTS AND HEMOGLOBIN

RED BLOOD CELL CHANGES

Some Conditions With High Red Blood Cell Counts

Physiologic.—

- Adrenaline excretion in certain emotional conditions
- Compensatory at times in anemia (chlorosis) and in leukemia under x-ray treatment
- Certain drugs
- Going from a low to a high elevation
- Exercise with excessive perspiration
- Hormone secretion
- Newborn
- Lowered oxygen tension
- Stress polycythemia

Pathologic.—

Acidosis	Chronic heart disease
Anoxemia	with cyanosis
Apoplexy	Congenital heart disease
Atherosclerosis	Diarrhea
Asthma	Emphysema
Bronchitis	Hemoconcentration, leading to shock
Carbon monoxide poisoning	Nephritis, rare
Carcinoma of the esophagus	Perspiration, abnormal
Cholera	Pneumothorax

⁷Rosenthal, N.: *J. Lab. & Clin. Med.* 13: 303, 1928.

⁸Schilling, V.: *Handbuch der normalen und pathologischen Physiologie*, Berlin, 1931, Springer.

⁹Wright: *Boston M. & S. J.* 154: 1906, and *Brit. M. J.*, 1906.

Poisons, some bacterial and chemical
 Polycythemia vera
 Pulmonary disease, chronic fibrotic
 Splenic tuberculosis
 Splenomegaly
 Suffocation

Trichinosis, at times
 Ulcer of pylorus, with vomiting
 Vomiting
 X-ray exposure in moderate doses,
 at times

Some Conditions With Low Red Blood Cell Counts

Physiologic.—

Temporarily following injections of fluids

Pathologic.—

All anemias and conditions accompanied by anemia, in varying degrees

HEMOGLOBIN CHANGES

Physiologic Increase.—

Changing from a low to a high altitude

At birth—after the fourth day it drops to 58 to 75% for the first year, then rises until it is normal at the fifteenth year

Physiologic Decrease.—

Temporarily after intravenous injections of large amounts of fluid

Premature children as low as 52% during the fifth month of life

Pathologic Conditions With Normal Hemoglobin.—

Often in agranulocytosis

Measles

German measles

Parotitis epidemica

Pseudoanemia Sahli

Varicella

Pathologic Conditions With Increased Hemoglobin.—

Chronic carbon monoxide poisoning
 and other conditions with
 hemoconcentration

Cardiac disease

Dehydrated states

Emphysema

Advanced empyema

Hemoconcentration leading to shock

Pathologic Conditions With Decreased Hemoglobin.—

Aleukemia

Amebiasis

Amyloid nephrosis

All anemias

Aplastic anemia

Arthritis

Banti's syndrome

Benzol poisoning

Chagas' disease

Chloroleukemia

Chlorosis

Clonorchis sinensis infestation

Coccidioidal granuloma

Diphyllobothrium latum infestation

Ectopic pregnancy

Egyptian anemia

Erythroblastic anemia

Fasciola hepatica—massive infestation

Gaucher's disease

Goat's milk anemia

Hematoxic anemias

Hemolytic anemias

Acute hemorrhage

Hodgkin's disease

Hookworm disease

Idiopathic hypochromic anemia

Progressive hypocythemia

von Jaksch's disease

Lead poisoning

Leishmaniasis (kala azar)

Leprosy

Leukemia

Acute leukemia of children

Chronic leukemia of children

Malaria

Niemann-Pick disease

Ovarian abscess

Oxyuriasis, untreated

Paratyphoid

Pernicious anemia

Acute febrile pleiochromic anemia
 of Moschcowitz

Pneumonia—at times slight increase

Poisoning with:

Acetanilid
 Acetphenetidin
 Other aniline
 derivatives
 Purine
 Potassium chlorate
 Pseudochlorosis infantum
 Pyelitis, chronic
 Raillietinosis
 Scarlet fever
Schistosoma haematobium infestation

Scurvy

Sickle cell anemia and other
 constitutional anemias
 Sprue
 Syphilis and anemia
 Syphilis, congenital
Taenia saginata infestation
 Trichinosis
 Tumors, malignant
 Trypanosomiasis
 Typhus
 Ulcerative colitis

WHITE BLOOD CELL CHANGES

Some Conditions With High White Counts

Physiologic.—

Afternoon counts are higher than morning counts
 Birth
 Digestion
 Exercise, including crying of infants
 Menstruation
 Pregnancy

Pathologic.—

Most acute bacterial infections, including those with abscess formation, almost anywhere in the body, one exception being the normal leukocyte count that usually attends brain abscesses

Trauma of all kinds including shock, hemorrhage, surgery, bone fractures, severe burns

Exposure to drugs and chemicals: Adrenalin, mercury bichloride, venoms of animals such as insect bites, black widow spider bites, and snake bites

Metabolic diseases such as diabetic coma, the nephritides, scurvy

Death of tissue such as occurs in infarction of organs including coronary occlusion or thrombosis, and embolism of any organ of the body, including the extremities

Mesenteric thrombosis is characteristically associated with a marked hyperleukocytosis.

Bleeding into organs or body spaces, such as hemorrhage into ovarian cysts, hemorrhage into the kidneys, or hemorrhage into the pleural or peritoneal cavity

Malignant tumors with marked degenerative changes

Any condition in which intravascular hemolysis occurs, such as in severe transfusion reactions due to incompatibility of blood, crises in sickle cell anemia, fatal blackwater fever

Protozoal diseases during attacks, such as malaria during the chill, congenital syphilis

Parasitic infestation

Some blood dyscrasias, such as polycythemia vera and leukemias

Some Conditions With Low White Counts

Physiologic.—

Injections of fluids
 Neurasthenia

Pathologic.—

Anaphylactic shock
 Avitaminosis, at times
 Banti's syndrome
 Basedow's disease
 Beriberi

Drugs, such as benzol, arsenic, lead at times, mercury, sulfa drugs, amidopyrine, urethane, nitrogen mustards, 6-mercapto-purine, gold salts, chloramphenicol, thiouracils

Empyema, where many leukocytes have poured into the area

Felty's syndrome

Food allergies

Gaucher's disease

Granulomata at times, sarcoid

Infections, bacterial, such as typhoid fever and some cases of brucellosis (Paratyphoid is usually accompanied by a normal or slightly elevated white blood count.)

Infections, viral, such as influenza, dengue, infectious mononucleosis (questionable virus), measles, pappataci fever, epidemic parotitis, poliomyelitis, vaccination, smallpox, scrub typhus or tsutsugamushi fever, infectious hepatitis.

Infestation, protozoal, such as disseminated histoplasmosis, kala azar, malaria, trypanosomiasis, yellow fever

Overwhelming infection, especially of the debilitated and of elderly people. This may be sepsis due to bacterial organisms of an acute type or may be associated with widespread disease such as miliary disseminated tuberculosis.

Hematologic disorders, such as agranulocytosis, "aleukemic" leukemia, aplastic anemia, infectious mononucleosis, pernicious anemia

Irradiation, either in the form of products of fission, such as the atomic bomb or radioactive isotopes, or exposure to x-ray

Myxedema

Osteoscleroses, diffuse

Paroxysmal hemoglobinuria, during attacks

Pellagra

Splenomegalic diseases, other than those listed here manifesting hypersplenism

Primary splenic neutropenia

Sprue

Vaccination, occasionally

Yellow fever at times

BLOOD PLATELET COUNT CHANGES

The normal number of blood platelets is usually from 250,000 to 300,000 per cu.mm. The highest number recorded is 2,220,000¹ in a case of polycythemia. The count is elevated by hunger and lowered by eating. Isaacs and Gordon² observed in Marathon runners three- to fivefold increases in the blood platelets after a race. Males show more platelets than females. Children show normal figures; but in senility, with bone marrow atrophy, the count is decreased. Menstruation is accompanied by a decline. Pregnancy figures show thrombocytopenia. Drugs influence the count materially. Adrenalin increases the number of platelets, as do pyrocin and hypotonic saline and sodium nucleinate by mouth. Injections of arsphenamine are followed by a decline in platelets, which also occurs after the administration of calcium, atropine, saponin, antimony, corpus luteum extract, tuberculin, and other substances.

Trauma may be followed by a decreased count. The count is normal in nephroses and lowered in uremic states, in jaundice, and in diseases of the liver generally. In kala-azar, there is a decreased blood platelet count. In acute infectious diseases, during the incubation period, the count is lowered, but with the febrile period the count rises. In the so-called Plaut-Vincent angina,

¹Epstein and Kretz: *Wien. klin. Wchnschr.* 9: 1177, 1930.

²Isaacs and Gordon: *Am. J. Physiol.* 71: 106, 1924.

there is a thrombocytosis. After administration of diphtheria antitoxin, there is a thrombocytosis. There is an increased count in typhus. In the acute exanthemata there is an increased count in the febrile period, particularly in measles, but in scarlet fever there is a decreased count. In tuberculosis it is claimed that the number of platelets is in direct proportion to the resistance of the patient; the higher the resistance, the greater the count. There is a decrease of blood platelets in aplastic anemia and in that form of purpura known as purpura hemorrhagica. There is also a decrease in acute leukemia. Gaucher's disease shows a decrease in blood platelets.¹ In acute lymphadenoses and lymphatic reactions generally, there is a thrombopenia. Chronic lymphadenoses, as well as Banti's disease, show a normal or slightly decreased count.

In affections of the myelocytic system, there is in panmyelophthisis a regenerative anemia, decline in leukocytes, and thrombopenia. In panmyelosis, there is increased manufacture of red cells, leukocytes, and thrombocytes.

In pernicious anemia there is a leukopenia and a thrombopenia. In hemolytic icterus, there is a variable picture with normal, subnormal, and increased counts. In secondary anemia there are increased counts dependent upon the blood loss. Accepting Schilling's ideas as correct, there is an explanation of the marked increase of blood platelets in posthemorrhagic anemia, hemolytic anemia, polycythemia, hemophilia, etc.

In chronic myeloid disease there is an increased count. In agranulocytosis, the count is normal.

For hemorrhagic diseases, refer to pages 905 ff.

Attention has been called in the literature to the relationship of the blood platelet count to postoperative venous thrombosis and postparturient venous thrombosis. Minot² and Sabin³ claim to have found megakaryocytes in the circulating blood of myelocytic leukemia in which condition there may be a very high blood platelet count. Cole,⁴ Ledingham and Bedson,⁵ and Robertson and others⁶ proved that the platelets act as a specific antigen and give rise to a specific antiserum. The presence of blood platelets in the earliest stage of clotting within the vessels has been shown by Bizzozero,⁷ Welch⁸ and Aschoff.⁹ Many have believed that platelets are intimately concerned with the coagulation of the blood, whether within or without closed vessels. Dawbarn, Earlam, and Evans¹⁰ examined cases of purpura hemorrhagica, pernicious anemia, acute leukemia, aplastic anemia, splenic anemia, and von Jaksch's anemia. Some of these cases had been splenectomized and blood platelet count and coagulation time were ascertained. They found that in cases with a low blood platelet count below a certain level (100,000) there was a slight delay in the clotting time as compared with that of the normal control. There was also a marked failure of clot retraction, much more parallel to the platelet counts than was

¹Keisman, M.: *Med. Klin.* 17: 72, 1921.

²Minot, G. R.: *J. Exper. Med.* 36: 1, 1922.

³Sabin, F. R.: *Bull. Johns Hopkins Hosp.* 34: 277, 1923.

⁴Cole, R. I.: *Bull. Johns Hopkins Hosp.* 18: 261, 1907.

⁵Ledingham and Bedson, S. P.: *J. Path. & Bact.* 31, 1928.

⁶Robertson, W. E., Illman, G. M., and Duncan, H. A.: *J. A. M. A.* 1: 1583, 1908.

⁷Bizzozero, J.: *Virchows Arch.* 1882, sc. 261.

⁸Welch, W. H.: *Albutt and Rolleston's System of Medicine*, ed. 2, London, 1910, 1, 691.

⁹Aschoff, L.: *Lectures on Pathology*, New York, 1924, p. 253.

¹⁰Dawbarn, R. Y., Earlam, F., and Evans, W. H.: *J. Path. & Bact.* 31: 833-873, 1928.

the coagulation time. In some cases of purpura, the platelets fluctuated normally, and clotting time and clot retraction were normal. They showed that after surgical operations there was a postoperative rise in blood platelets, first manifested on the sixth day and reaching a maximum in about ten days. The rise persisted for a few more days. Observations made in the third week and onward showed normal counts. The degree of rise was apparently related to the severity of the operation but varied in different patients subjected to the same operation. They noted a diminution of the blood platelets in association with an increase of blood coagulation time, and an excess of platelets with a shortening of the blood coagulation time. In purpura hemorrhagica, their counts of platelets ran as low as 18,000 and as high as 287,000; in pernicious anemia, from 34,000 to 110,000; in acute leukemia, from 25,000 to 90,000; in splenic anemia, from 42,000 to 232,000; in anaphylactoid purpura, from 374,000 to 724,000; and in hemophilia, from 303,000 to 713,000. Hueck,¹¹ studying one hundred cases after surgical operations, showed a slight fall in blood platelets during the first few days after operation, followed toward the end of the first week by a rise which reaches its maximum about the tenth or twelfth day and then returns to normal. Brock and Long¹² reported a case of left-sided phrenic evulsion with a slight febrile reaction accompanied by a slight rise in the platelet count. The first stage of a posterior, extrapleural thoracoplasty was performed, followed by an *initial* fall, followed by a *rise* commencing about the *seventh* day, and reached its *maximum* on the *thirteenth* day. On the *sixteenth* day the count was 448,800 per cu. mm., when the patient noticed stiffness in the left thigh and calf, pain in the leg, and a small, firm clot could be palpated quite easily over the great saphenous vein in the middle of the thigh. The platelet count fell and the thrombosis followed a normal course without embolism. Brock suggests a possible line of treatment to prevent thrombosis; namely, to find a substance or means which would prevent or diminish the normal postoperative increase in platelets.

Gradwohl and Hiller's¹³ investigations on this subject were as follows: Forty cases at the St. Louis County Hospital, including 12 herniotomies, 4 miscellaneous operations, 1 hemorrhoidectomy, 2 cholecystectomies, 14 pregnancies, and 2 normals, were studied. Platelet counts were made from the first to the fifteenth postoperative day. After herniotomies, blood platelet counts rose from as low a count as 150,000 to as high a count as 850,000 in one case and 800,000 in another case. In a third case, Case 9, of the series, the count rose to 920,000.

Fourteen obstetrical cases were studied with blood platelets from 250,000 to 600,000. Hysterectomies had blood platelet counts ranging from 250,000 up to as high as 800,000. Cholecystectomies ranged from 275,000 to 1,170,000. The four miscellaneous cases showed counts from 300,000 to 538,000.

Two normal individuals were studied daily and ran counts from 209,000 to 329,000 on the tenth day, and from 213,000 on the first day to 207,000 on the tenth day.

¹¹Hueck, H.: München. med. Wehnschr. 73: 173, 1926.

¹²Brock, R. C., and Long, M. S.: Lancet 1: No. 5718, p. 688-690, 1933.

¹³Gradwohl, R. B. H., and Hiller, S. J.: J. Missouri M. A. 31: 392, 1934.

From these investigations, it is evident that there is very little change in the blood platelet count from day to day in the normal individual. It is also evident that after certain operations, such as herniotomy, there is a definite increase. After hysterectomy, there is also an increase. There is uniformity in the rise of blood platelets after herniotomies. Since postoperative venous thrombosis seems to occur at times following a herniotomy, the rise in blood platelets in this condition may have some relationship to this accident. However, the fact that we have rises in blood platelets in the post-operative periods of other conditions makes us give pause in drawing conclusions about this possibility.

In the hemorrhagic diatheses, a marked decrease in blood platelets is seen in these purpuric individuals. When the blood platelets fall below 35,000 per cu. mm., there is usually spontaneous hemorrhage into the tissue, but *thrombocytopenia* may be followed by neither hemorrhage nor purpura. Refer to pages 905 ff. for a complete discussion of this subject.

Summary of Changes in Blood Platelets

Physiologic Increase

- At birth (not constant)
- Marathon runners
- After administration of certain medicaments; for example:
 - Diphtheria antitoxin
 - Adrenalin
 - Pyrocin
 - Hypotonic saline and sodium nucleinate by mouth
- After operations (not constant)
- Blood which is shut off from circulation in a doubly ligated vein

Physiologic Decrease

- After eating
- After administration of certain drugs; for example:

Arsphenamine	Antimony
Calcium	Corpus luteum extract
Atropine	Tuberculin
Saponin	Phenylhydrazine
- Menstruation
- Pregnancy
- Senility

Increase in Disease

- Bubonic plague
- Often in chronic hemolytic icterus
- Febrile period of acute infections
- Hemolytic anemia
- Chronic myeloid disease
- Pneumonia
- Polycythemia vera
- Posthemorrhagic anemia
- Sickle cell anemia
- Typhus

Variable

- Hemolytic icterus
- Secondary anemias, depending on blood loss

Normal Counts in Disease

Agranulocytosis
 Athrombocytopenic purpura
 Banti's syndrome (they may be decreased in this condition)
 Chlorosis
 Nephrosis

Decrease in Disease

Aleukemic myelosis	Acute lymphadenoses
Idiopathic aplastic anemia	Lymphocytic leukemia
Benzol poisoning	Panmyelophthisis
Acute infections during the incubation periods	Pernicious anemia
Jaundice	Purpura, thrombocytopenic
Kala-azar	Scarlet fever
Acute leukemia	Trauma
Diseases of the liver	Uremia
	Varicella at the beginning of the fever

TECHNICS IN HEMATOLOGY**INTRODUCTION**

The arrangement of the material on technics in this revision is a departure from previous editions. The purely technical phases have been organized together, as a ready reference section for technics. However, it should be stressed that reading of the text related to the various elements in the blood is important for a more comprehensive understanding of the subjects involved, by physicians and medical students, and in the training of technicians.

Blood examinations are important in completing the examination of every patient. These tests must be performed with consummate technical skill, utilizing procedures and equipment best suited for various situations. This calls for intelligent planning based on many factors. The planning must begin with the patient-physician sphere of activity. Certain hematologic tests can be considered as basic routine "screening" procedures. These should include hematocrit and hemoglobin determinations, with examination of a properly prepared blood spread. A sedimentation rate may be included as routine if the Wintrobe hematocrit test is used. The results of these tests will often point the way to other special procedures, or the history and physical examination may indicate the need for more exhaustive hematologic investigation of the patient. In ordering tests or interpreting the results, knowledge of the normal ranges rather than some fixed number and the limitations of accuracy of the procedures is of inestimable value to the physician.

Sound planning in the laboratory implies a thorough knowledge of equipment and procedures which can be gained only by consistently keeping abreast of such matters by reading the literature and by attendance at meetings especially aimed in these directions. The purchase of equipment should be based on the work to be done, as well as on the accuracy of the instrument. For example, photoelectric hemoglobin methods lend themselves readily to assembly line organization, which makes them a real economy in technician time. At the same time, maximum accuracy can be obtained by use of a photometer. In a laboratory doing a large volume of work, such equipment

almost becomes a necessity. However, the less expensive visual hemoglobinometer may serve satisfactorily in a physician's private office if the volume of work is minimal. In either case, knowledge of the limitations of the equipment is necessary for proper appreciation of the results. An improperly standardized expensive photoelectric apparatus may be more inaccurate than less expensive equipment. In both instances, careless technic will militate against accuracy.

The choice of procedures and full knowledge of their limitations should be considered. Frequently, relatively simple procedures will suffice and thus eliminate more complicated and more time-consuming technics. The estimation of blood platelets is a good example of such a situation. In a properly prepared blood spread, if platelets are seen in every oil immersion field, it can be safely said that thrombocytopenia does not exist. In clinical conditions with a bleeding diathesis and obvious normal numbers of platelets in the spreads, the actual counting of platelets is unnecessarily time-consuming. On the other hand, if trends in numbers of platelets are to be determined, then platelet counts by more precise methods are in order. There may be situations in which simple or commonly performed tests may not reveal any abnormality and it might be necessary to perform some little known or infrequently used test to uncover the basic disorder. An example of such is the plasma thromboplastin component determination in a patient with a bleeding diathesis with normal routine test results.

These are but a few examples of intelligent choice of procedure indicated by the clinical state of the patient. The choice of technic may be influenced by the range of accuracy desired or desirable. Erythrocyte counting, for example, lends itself to scrutiny from this standpoint. The primary purpose of a red blood cell count is determination of the presence or absence of anemia or polycythemia. In routine work, significant deviations can be determined simply with an accuracy of ± 5 per cent for hemoglobin and ± 2 per cent for hematocrit. On the other hand, routine erythrocyte counts using 1 pipette, 1 chamber, and 500 red blood cells are subject to an inherent error of ± 16 per cent.^{1, 2} This means that a red blood count that is actually 5,000,000 per cu. mm. may be reported as 4,200,000 or 5,800,000 per cu. mm. at either extreme. These discrepancies in the limits of accuracy of this test should make it quite obvious as to the choice of tests for routine work, namely, the elimination of the red blood count. If the hematocrit and hemoglobin tests reveal subnormal values, then a well-made erythrocyte count is indicated. We heartily endorse Ham's² recommendation as to the minimum procedures for red blood cell counting for clinical use: 1 pipette, 2 chambers in the hemacytometer, and counting 400 to 600 cells per chamber. For determination of the corpuscular constants (mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration), Ham recommends using 2 pipettes, 2 chambers, and counting 400 to 600 red cells in each chamber.

It is of utmost importance that technicians present an appearance to the patient and to the physician that reflects confidence. Laboratory technicians

¹Berkson, J., Magath, R. B., and Hurn, M.: *Am. J. Physiol.* 128: 309, 1940.

²Ham, T. H.: *A Syllabus of Laboratory Examinations*, Cambridge, Mass., 1950, Harvard University Press, pp. 45-47.

should appear neat and clean; particularly is this true of the hands, which are often stained and generally untidy in appearance. Constant observation, reading, and the repeated performance of tests will aid in bestowing confidence in oneself, which will be reflected in the patient's response. It is important for technicians to become thoroughly familiar with all phases of every test that they perform and to know the limitations of the particular test that they are reporting. There is too great a tendency for some to believe that results obtained in many tests are rigidly accurate scientific figures. For this reason, in the following section on technic and in some of the preceding sections, a mean average and a range of the test are included. The standard values that follow have largely been taken from *The Handbook of Biological Data*, edited by E. C. Albritton.¹ This compendium of data was obtained by contributions "by experts in the various areas of hematology. These tables have been reviewed by outstanding specialists in the subjects covered and represent an unusually complete collection of data on blood, which is unique in the high degree of reliability. Over six hundred leading investigators in biology and clinical science were consulted for the accumulation of these data. In addition, numerous outstanding experts reviewed the material prior to publication for its accuracy."

The one procedure common to all hematologic tests is the drawing of blood samples from patients. This procedure must at all times be carried out with full knowledge and appreciation of asepsis. *All materials which touch the patient for obtaining a blood specimen must be sterile.* This means sterile needles, cotton, and syringes. The skin must be thoroughly cleansed with some antiseptic, alcohol, alcohol-ether mixture, acetone, Merthiolate, Zephiran, or others. After a skin area has been cleansed, the operator must not touch the area with the fingers. It is too common a practice to locate a vein in an arm, cleanse the area, and then proceed to palpate the area again with the nonsterilized finger before inserting the needle. *It must be repeated that if the finger touches the area to be punctured, this area must be cleansed again.*

Instruments Used to Puncture the Skin.—Any instrument used to puncture the patient's skin for the purpose of obtaining blood for examination must be absolutely sterile. *This does not mean that one may use a needle attached to a cork which is carried point downward into a small bottle containing alcohol or any other chemical or disinfecting liquid.* Sterility means just what it implies, namely, actual killing of bacteria or viruses. It is well known that cases of infectious hepatitis, for instance, have occurred in hospitals where imperfectly sterilized needles and lancets have been used. All instruments must have been sterilized by dry heat at 170° C. for 1½ or 2 hours.

There are four different methods suggested: (1) Break sharp-pointed pens in half, wrap a number in paper, and sterilize by dry heat at 170° C. for 1½ to 2 hours. (2) Use Hemolets² (Fig. 143). Hemolets are razor-sharp ribbons of stainless steel packaged in sterile wrappers. They are thrown away after being used once. (3) Use Sera-Sharp² sterile disposable lancets (Fig.

¹Albritton, E. C.: *Standard Values in Blood*, Wakefield, Mass., 1952, MacGregor & Werner, Inc.; republished Philadelphia, 1952, W. B. Saunders Co.

²Procurable from Scientific Products Division, American Hospital Supply Corporation, 1210 Leon Place, Evanston, Ill.

144). These also are used once and discarded. They are inexpensive and need not be used more than once. By using Hemolets or Sera-Sharp, the possibility of transmitting infectious diseases such as virus hepatitis,* homologous serum jaundice, malaria, etc., through skin puncture can be precluded. Both the Hemolet and the Sera-Sharp make the puncture about the correct depth, not too shallow and not too deep. (4) Use the Steri-Lance¹ (Fig. 145).

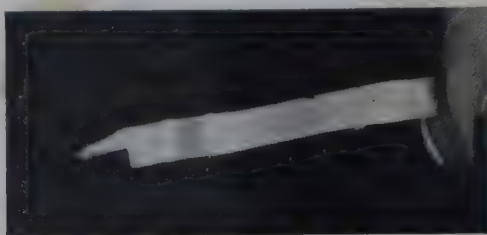


Fig. 143.—Hemolet. (Courtesy American Hospital Supply Corporation, Chicago, Ill.)



Fig. 144.—Sera-Sharp. (Courtesy Propper Manufacturing Company, Inc., Long Island City, N. Y.)



Fig. 145.—Steri-Lance, an adjustable automatic lancet for skin puncture. (Courtesy Propper Manufacturing Co., Inc., Long Island City, N. Y.)

*See pages 1185 and 1725.

¹Procurable from Propper Mfg. Co., Long Island, N. Y.

To use the Steri-Lance, place all lancet heads into the side of the container marked "sterile." Place the lancet holder in the center groove. Sterilize the complete unit at 15 pounds pressure for 30 minutes in an autoclave.

To attach the blade unit, slip the end of the holder over a sterile lancet head and screw on securely with a few turns of the holder. Holding the assembled lancet with the blade up, turn the screw cap of the opposite end until the tip of the blade is just barely visible. Once adjusted, the holder will give a uniform depth of penetration with all blades. The lancet blade is not attached to the spring, but has a special recoil action. Pull out the top cap of the holder until the spring catches, place the tip of the lancet blade against the disinfected skin in the usual manner, and press the release level.

After use, wipe the blade clean, then place the lancet head in a pocket on the unmarked side of the insert, and unscrew by twisting the handle. *It is never necessary to touch the lancet blade or head at any time.* After all the blade units have been used, the complete unit is ready for sterilization. To prevent confusion, do not transfer blade units from "unsterile" to "sterile" side until they are ready for sterilization.

Technic of the Puncture.—To use the sterile pen point, Hemolet, Sera-Sharp, or Steri-Lance, hold the lobe of the ear between the thumb and forefinger.

Place the puncture point on the lobe of the ear, and make a quick incision. If the hand is used, hold the hand tight before making a "jabbing" puncture. Blood should ooze in small drops spontaneously, or with very slight pressure. *Do not use strong pressure to increase bleeding.* Such pressure causes the blood films to become contaminated with tissue juice and endothelial elements, and alters the patient's counts.

Remove the first small drop of blood, then proceed with the various counts and blood spreads. When finished, wipe the skin with alcohol and discard the used cotton.

Proper types of blood samples are important; their source is often dependent upon the nature of the test to be performed. Some routine hematologic procedures such as red blood count, white blood count, hemoglobin, and blood spread, can be obtained from capillary blood taken by simple skin puncture, as directed above.

Blood specimens for many hematologic procedures are today obtained in many hospitals by venepuncture.*

To Use Venous Blood.—The method of making a venepuncture of an adult is given on page 2031.

Venous blood must be transferred quickly to a suitable anticoagulant to prevent clotting. A satisfactory anticoagulant mixture, known as "3:2 oxalate" mixture is prepared as follows:

3:2 Oxalate Mixture

Ammonium oxalate	-----	1.2 gm.
Potassium oxalate	-----	0.8 gm.
Distilled water	-----	100.0 c.c.

Use 0.1 c.c. for each c.c. of blood after the anticoagulant has been dried. For routine work, place 0.5 c.c. into test tubes or suitable wide-mouth bottles.

Allow to dry or heat gently in an oven to evaporate the water, which leaves a white, dry powder on the bottom of the collecting vessels. Stopper and keep away from dust.

Place the blood into the anticoagulant tube and invert gently 5 to 10 or 20 times to insure adequate mixing of the anticoagulant and blood.

Venepuncture in Infants.—

To obtain a large sample of venous blood from an infant, recourse must be had to puncture of the femoral vein or puncture of the fontanel. Both procedures are relatively formidable and should be undertaken only by a physician, and not by a technician.

*The author (R. B. H. G.) still prefers the use of peripheral blood obtained by skin puncture.

For small quantities of blood, one can easily obtain samples by puncture of the baby's thumb, large toe, or heel. This should be done under strict asepsis, namely, cleansing of the skin and of the puncture points with an antiseptic soap and water, or by saline, and then one of the skin antiseptics, such as a 1:1,000 solution of tincture of Merthiolate. After this is applied, and allowed to dry, punctures are made with an extremely sharp-pointed surgical knife. One with a long, tapered point is preferred. When making several of these punctures, and constricting the ankle, if the heel or toe is used, enough blood can be obtained to carry out a serologic procedure.

Blood kept in the anticoagulant may be used for making erythrocyte counts, hemoglobin, white blood counts, sedimentation rates, hematocrit determinations, fragility tests, reticulocyte counts, icterus index, and other tests, if performed within three to four hours after collection of the blood sample. However, the author (R.B.H.G.) prefers that blood for hematologic investigations be taken by skin puncture.

Blood taken in an anticoagulant is generally unsatisfactory for the preparation of blood films because both the leukocytes and the red blood cells show morphologic alterations that render cell identification difficult at times. With a little practice, it is possible to take a small drop of blood from the needle at the time of venepuncture, place it on a glass slide, and make a satisfactory blood spread.

Precautions to Be Observed in Handling Blood for Hematologic Study.—The blood sample should be kept away from excessive heat, such as radiator under a laboratory table, or an oven, since heat will hemolyze the blood.

Thorough mixing of the blood specimen prior to removal of samples for each examination is necessary to secure true sampling of the specimen.

One advantage of collecting blood routinely in an anticoagulant is that tests may be repeated and extra procedures carried out if indicated without recalling the patient and repeating the venepuncture. Another advantage is the multiple number of tests which may be performed without concern about clotting of blood before pipetting has been done, or closing of the puncture wound before sufficient specimen has been collected.

Glassware must be thoroughly cleaned and dried. Immediately after using, the pipettes, counting chambers, hematocrit tubes, etc., must be cleansed with cold water to prevent staining the glassware with dried blood. If these instruments become brown, they must be treated with strong acids or ammonium hydroxide, which is undesirable.

Slides and coverslips used for preparing blood spreads must be new and not re-used, and they should be cleaned thoroughly. Ordinary glass slides may be cleaned readily by the vigorous use of clean dry gauze. The fingers must not touch the edges of the slide nor the slide surfaces.

The care and use of the microscope are discussed in Chapter I. For oil immersion work, maximum light intensity with the condenser in the closest position to the blood film and the light shutter open to its maximum will give the clearest picture of the fields. Usually a combination of both the light shutter and the condenser position are required to obtain maximum benefit for examination of various types of material.

Reporting results of laboratory tests is an important function of the laboratory. Even the most meticulous technic requiring many hours of time

would be worthless if the results were not recorded in some proper manner. Reports should display normal values, ranges for normal values, as well as the result of a particular test. Duplicates of reports should be retained by the laboratory for future reference. Promptness in reporting will gain the plaudits of the referring physician. Reports on examination of blood films should be so worded that the referring physician can visualize the film. Laboratory reports constitute one link in the chain of confidential relationship between patient and physician. Laboratory technicians have been taken into this confidence. *Under no circumstances should they betray this confidence by revealing reports to persons unauthorized to receive such information.*

All material, such as blood specimens and reagents, must be properly labeled in order to avoid both confusion and error. Labeling of blood should be performed at the time the material is collected. Bottles or test tubes may be labeled either with wax pencil or with some type of adherent label suitable for inscription. Blood slides may be labeled very readily by writing across the thick part in pencil; another method is to scratch the identification with the puncture point, in very small letters, across the beginning end of the blood film. In such manner, a permanent label is obtained. Individual pipettes may be labeled with special tubing, or the laboratory request slips may be attached to the pipettes.

NUMERICAL COUNTING OF ERYTHROCYTES AND LEUKOCYTES

Numerical Counts of Erythrocytes

Normal values of erythrocytes are:

Males: 4,600,000 to 6,200,000 average 5,400,000 per cu. mm.

Females: 4,200,000 to 5,400,000, average 4,800,000 per cu. mm.

The normal values in children are somewhat different.

Equipment and Reagents.—

The blood specimen may be capillary or venous oxalated blood.

Red Blood Cell Diluting Pipette.—There are two types of red counting pipettes, the standard Thoma and the Trenner. The Thoma pipette illustrated in Fig. 152 has 10 divisions on the capillary end, with 0.5 and 1.0 points numbered prominently. If blood is drawn to the 0.5 mark and the pipette filled with diluting fluid, the resultant dilution is 1:200 (1 volume of the 101 total remains in the stem, does not enter the bulb, and is blown out first before the counting chamber is filled, and therefore does not dilute the blood). This is the dilution used in routine counting. If the patient is quite anemic, a lesser dilution may be made by drawing the blood to the 1.0 mark and then filling the pipette with diluting fluid, a dilution of 1:100. Obviously, various dilutions may be obtained by using the different divisions on the capillary or stem for measuring the amount of blood to be used.

The Trenner or “automatic” pipette is so constructed that when blood is drawn into the capillary end it will be carried by capillary attraction to a given point in the capillary. These pipettes permit only one dilution, 1:200.

Diluting Fluid.—

a. Hayem Red Cell Counting Solution.—

Mercuric chloride, c.p. -----	0.5 gm.
Sodium sulphate, c.p., crystalline -----	5.0 gm.
or anhydrous -----	2.65 gm.
Sodium chloride, c.p. -----	1.0 gm.
Distilled water -----	200.0 c.c.

Dissolve all the chemicals in the distilled water and filter several times through the same filter paper. Make a sufficient supply for only two or three weeks, since deterioration might occur beyond this time, although Hayem solution is fairly stable. If a precipitate forms, discard the solution. This solution must be used at room temperature to prevent clumping of the erythrocytes due to the cold. Clumping of the erythrocytes may also occur in diseases associated with hyperglobulinemia, such as multiple myeloma. Gradwohl has observed this phenomenon in Hodgkin's disease. It has been recommended that 0.01 gm. of gelatin be added to each 100 c.c. of solution to prevent such agglutination.¹

b. Gower Diluting Fluid.—

Glacial acetic acid	16.65 gm.
Sodium sulphate, c.p., crystalline	6.25 gm.
Distilled water	100.0 c.c.

Add the ingredients slowly to the distilled water and mix thoroughly. It has been claimed² that Gower solution prevents rouleaux formation of the erythrocytes.

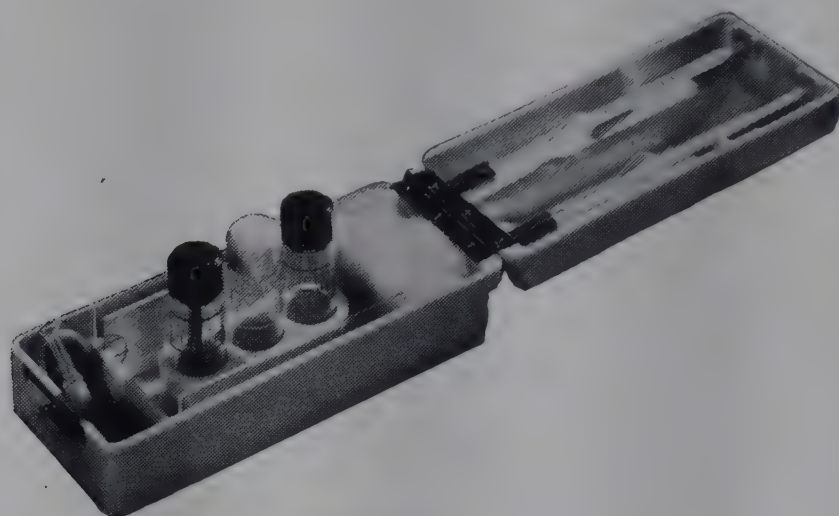


Fig. 146.—Bard-Parker hematologic case.

Hemocytometer.—

A counting chamber basically consists of a glass plate upon which have been etched lines, permitting accurate measurement of small volumes of blood. Fig. 148 illustrates one such hemocytometer. Fig. 151 shows the ruling of a single counting chamber. The most commonly used ruling is the improved Neubauer (5 in Fig. 150). This ruling has been reproduced in Fig. 153. Note that a single chamber (of which there are usually two per hemocytometer) consists of 9 large squares, each measuring 1 sq. mm. Each of these squares covers a low-power field (16 mm. objective, 10× ocular). The central square of the chamber is subdivided into 25 smaller squares (each $\frac{1}{25}$ sq. mm.), 5 of which are marked "R" in Fig. 153. Each of these 25 squares is further divided into 16 squares. These 5 squares, marked "R," are used for routine erythrocyte counting. A magnification of one of these "R" squares, containing erythrocytes, is illustrated in Fig. 151. One of these $\frac{1}{25}$ sq. mm. fields covers a high dry (4 mm. objective, 10× ocular) field.

Formula for determining factors in hemacytometry:

$$V = D \times Dp \times S.A$$

V is the volume of blood actually measured.

D is the dilution of the blood sample (variable).

Dp is the depth of the counting chamber (always $\frac{1}{10}$ mm.).

S.A is the surface area counted (variable).

The formula for correcting counts to the number of cells per cu. mm. of whole blood is

$$C = N \times \frac{1}{V} \text{ per cu. mm.}$$

¹Tompkins, E.: J. Lab. & Clin. Med. 33: 480, 1948.

²Ch'u, Y., and Forkner, C. E.: J. Lab. & Clin. Med. 13: 1282, 1938.

C is the cell count per cu. mm.

N is the number of cells actually counted in the chamber.

V is the value from the formula above.

An example of using these formulae is given on pages 574, 575, 576, 577.

These formulae are applicable to any cell counting performed in a hemacytometer.

Hemacytometer Cover Glass.—This is especially designed for this purpose. Do not substitute ordinary laboratory cover slips for these.

Microscope.—

Carrying Case for Hematologic Outfit.—It is frequently necessary to collect blood for red and white counts outside the laboratory, hospital, or physician's office. Under these circumstances, the use of the Bard-Parker hematologic case,* or some similar device, is recommended. See Fig. 146.

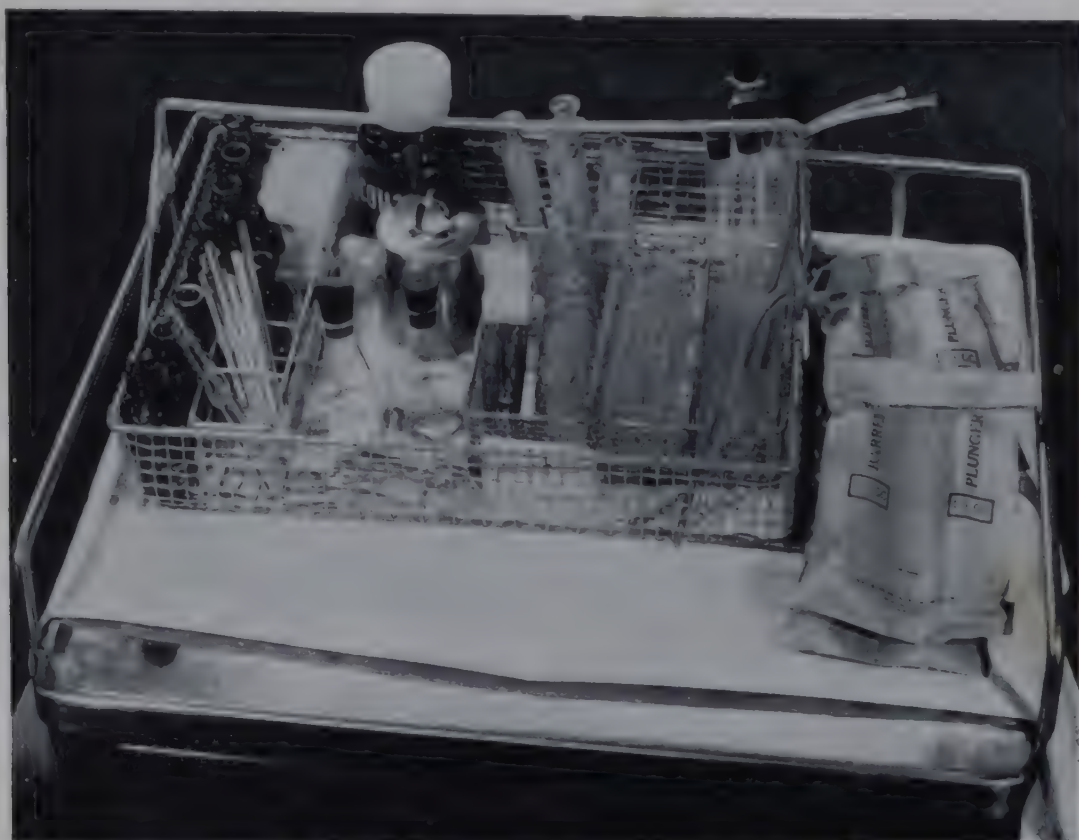


Fig. 147.—Hospital blood procurement tray. Materials are carried on a suitable cart with wheels. Wire carrying basket (courtesy A. S. Aloe Co., St. Louis, Mo.). "Duets" sterile wrapping containers for syringes (courtesy Central States Paper and Bag Co., St. Louis, Mo.). Hemolets for finger puncture, sterile needles, cotton balls and antiseptic, and appropriate containers for blood specimens, pipettes for individual blood samples (courtesy Veterans Hospital and Jewish Hospital, St. Louis, Mo.).

Considerations Preparatory to Making Counts

1. Counts should be taken in the morning while the patient is still in bed. Violent movement, work, or exercise may provoke temporary pictures which are not normal. Food or prolonged fasting usually increases the count. If consecutive counts are made, they should be made at the same hour each day.

2. Assemble the following material.

Sterile puncture point.

Ether and alcohol.

Red and white counting pipettes, which should be dry.

Hayem red counting solution and white counting solution, previously filtered.

Small tubes, or watch glasses, for holding the counting solutions.

*Manufactured by Bard-Parker Co., Inc., Danbury, Conn.

Preparation of the Patient.—Cleanse the ear lobe with ether, to produce hyperemia. If alcohol is used, it may cause the cells to change shape. Ether cleanses and at the same time produces hyperemia. If the finger is to be used, produce hyperemia by immersing the entire hand in a basin of hot water. Dry the hand and disinfect the skin with ether or alcohol and dry. If venous blood is used, transfer to special anticoagulant, and mix thoroughly to prevent clotting.

Technic of the Puncture.—This is explained on page 567.

Taking the Red Count.—

If capillary blood is obtained from skin puncture, wipe off the first drop with a piece of dry sterile cotton, and use successive drops obtained without undue squeezing of the part, working rapidly to prevent clotting of the blood. If oxalated blood is used, it must be thoroughly mixed prior to use.

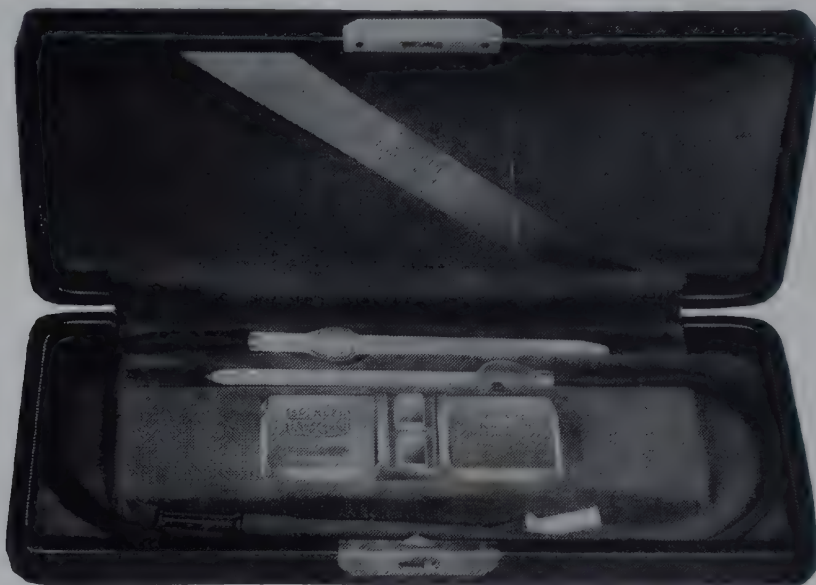


Fig. 148.—Complete hemacytometer including improved double Neubauer counting chamber. (Courtesy American Optical Company, Scientific Instrument Division.)

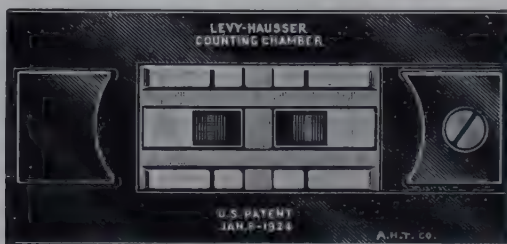


Fig. 149.—The Levy-Hausser counting chamber mounted in a molded bakelite holder, forming a practically unbreakable unit. (Courtesy Arthur H. Thomas Co.)

Make the dilutions of blood as perfectly as possible.

Holding the pipette up at an angle of 45 degrees in the drop of blood, and not touching the skin, draw the blood to exactly the 0.5 mark of the counting pipette (for routine counts). Always draw the blood into the pipette; never allow it to run in by capillary attraction.

Wipe the tip of the pipette clean of blood with a piece of dry cotton, gauze, or filter paper, without touching the opening of the capillary, and then immerse in the freshly filtered diluting fluid, which should be in a small tube or a watch glass. Do not insert the pipette in a bottle of counting solution.

Draw the diluting fluid steadily into the pipette by gentle mouth suction to exactly the 101 mark past the bulb of the pipette, rotating the pipette on its long axis to insure thorough mixing of blood and diluent, and gradually getting the pipette into a vertical position so that the measurement at the top of the pipette may be accurate. Do not permit the capillary tip of the pipette to come out of the diluting fluid while drawing up the solution, and do not permit any of the blood to run out of the capillary while filling

the pipette. If the diluting fluid has been permitted to go beyond the 101 mark, or if air bubbles are present in the pipette, discard the mixture and begin again with a clean, dry pipette.

Immediately mix the contents of the pipette thoroughly by placing the thumb over one end and the first finger over the other end, and shaking for a minute or so.

The diluted blood may be transported to the laboratory for further examination. Take care that none of the fluid escapes from the pipette. Lay the pipette on its side and avoid touching the tip to any object. Various devices, including wide rubber bands and pipette tubing, have been used for sealing the pipette. Pipette closures are available commercially. If rubber bands are used, seal off the small capillary end of the pipette first, to prevent expulsion of fluid. In removing the device, reverse the procedure. When picking up the pipettes for manual shaking, follow the same order of holding the pipettes.

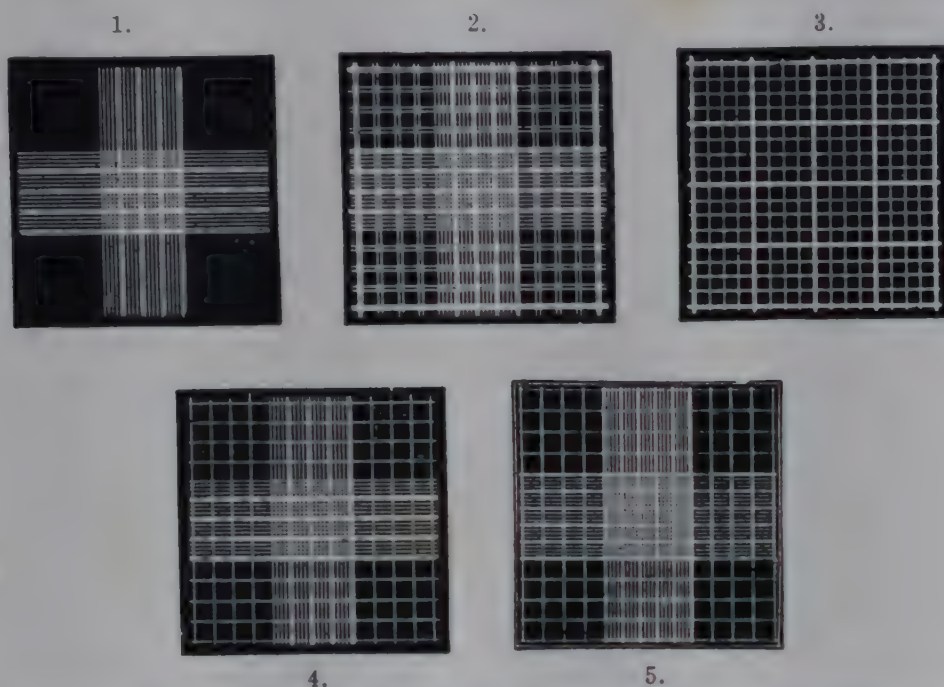


Fig. 150.—Hemacytometer counting chamber rulings: (1) Thoma; (2) Tuerk; (3) Fuchs-Rosenthal; (4) Neubauer; (5) Improved Neubauer. (Courtesy E. H. Sargent Co., Chicago.)

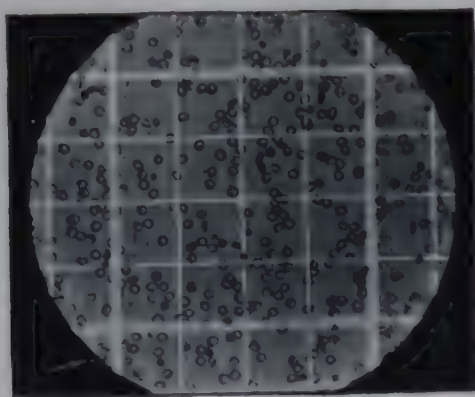


Fig. 151.—Bright line counting chamber. (Courtesy American Optical Co., Scientific Instrument Division.)

Diluted blood samples may be examined within two hours. However, these must be thoroughly mixed just prior to their admission into the hemacytometer.

Filling the Counting Chamber.—Shake the pipette for 2 or 3 minutes, holding it in one hand and striking it gently against the opposite hand. Mechanical shakers are available commercially and are highly desirable, particularly in a laboratory doing a large volume of work.

Thoroughly cleanse the hemacytometer and coverslip and render dry and fat-free. After use, wash in running cold water and wipe dry with fine gauze, lens paper, or linen. Avoid touching surfaces which are to come in contact with the blood specimen.

Place the coverslip in its proper position over the ruled areas of the hemacytometer and examine the assembled instrument for cleanliness under the low power of the microscope.

Discard the first three or four drops of diluted blood from the pipette and then fill the counting chamber by capillary attraction, by placing the tip of the pipette at one of the open ends of the chamber. The angle of the pipette and the time required for filling the chamber vary with the speed of the pipette and can be determined only by experience. Carefully fill the counting chamber exactly. Overfilling can be readily observed by the presence of fluid in the moats surrounding each ruled area. If this occurs, clean the instrument and refill the counting chamber. An insufficient amount of fluid may be noted by the failure of fluid to cover a ruled area. Rectify by adding more fluid to the chamber. If air bubbles are present in the chamber, they usually indicate lack of cleanliness of the hemacytometer or coverslip or moisture. Clean again and refill the chamber.

Fill both ruled areas successively.

Allow the mixture to settle in the chamber. Locate the ruled area for erythrocyte counting under low magnification, then change to the high dry objective for actual counting of the cells.

Making the Count.—

In a routine erythrocyte count, enumerate the cells in the five "R" squares, Fig. 153. Since Hayem's solution does not dissolve leukocytes, do not count such cells in the red blood cell count. Leukocytes are larger than erythrocytes, appear grayish, and usually are granular. Only one such cell is seen in approximately 750 to 1,000 erythrocytes, and therefore leukocytes do not present a problem in routine red cell counting. When the white count is very high, particularly in leukemia, it is very important to make this differentiation.

A standardized pattern of counting should be adopted. It is recommended that counting begin in the left upper corner of each "R" square and proceed as follows: count the first row of small subdivisions to the right, drop down to the second row and count to the left, drop down to the third row and count across to the right, and then finish the fourth row counting to the left. In the enumeration, include all cells touching or half in and half out of the upper line and the line to the left, excluding those half in and half out of the lower and right-hand lines. Take care not to count any cell twice and do not omit any cells. Remember that the boundary line is the center line of the triple ruling. Count the first "R" square at the upper left of the ruling, the second at the upper right, the third at the lower right, the fourth at the lower left, and the fifth in the center. See Fig. 153.

Record the count in each "R" square, and obtain the sum of the cells in the five squares. (Mechanical counters are available commercially.)

Count two such chambers and take the average.

Calculation.—

Example for Routine Counting.—

Multiply the sum of the 5 squares by 10,000.

454 cells counted in 5 "R" squares, in 1 chamber.

456 cells counted in 5 "R" squares in the second chamber.

2) 910

455 cells counted in an average of 5 "R" squares.

Dilution of the blood 1:200. Area counted $\frac{5}{25}$ or $\frac{1}{5}$ sq. mm.

$V = \frac{1}{200} \times \frac{1}{10} \times \frac{1}{5} = \frac{1}{10,000}$ cu. mm.

$C = 455 \times \frac{1}{\frac{1}{10,000}}$

$= 455 \times 10,000$

$= 4,550,000$ erythrocytes per cu. mm. blood.

For Anemic Blood:

Blood was drawn to the mark "1" in the pipette, making the dilution of blood 1:100.

455 cells were counted in the 5 "R" squares.

$V = \frac{1}{100} \times \frac{1}{10} \times \frac{1}{5}$ cu. mm. $= \frac{1}{5,000}$ cu. mm.

$C = 455 \times \frac{1}{\frac{1}{5,000}} = 455 \times 5,000 = 2,275,000$ erythrocytes per cu. mm.

For Extremely Anemic Blood:

Blood was drawn to the mark "1" in the pipette, making the dilution of blood

1:100.

455 cells were counted in 10 "R" squares in each drop.

$$V = \frac{1}{100} \times \frac{1}{10} \times 100 = \frac{1}{100} \text{ cu. mm.}$$

$$C = 455 \times \frac{1}{\frac{1}{100}} = 455 \times 100 = 45,500 \text{ erythrocytes per cu. mm. blood.}$$

For Polycythemic Blood:

600 cells counted in 5 "R" squares.

Blood was drawn to the 1/2 mark in the pipette, making a dilution of 0.4:100 or 1:250.

$$V = \frac{1}{250} \times \frac{1}{10} \times 100 = \frac{1}{250} \text{ cu. mm.}$$

$$C = 600 \times \frac{1}{\frac{1}{250}} = 600 \times 250 = 150,000 \text{ erythrocytes per cu. mm. blood.}$$

During the counting process observe the distribution of the erythrocytes. If clumps of cells are seen, the distribution is unsatisfactory and an entirely new dilution of blood is required. If motion continues in the preparation in a streamlike fashion, counting is inaccurate. Such motion may result from overfilling or underfilling the chamber or from drafts caused by fans or open windows. These features must be corrected or eliminated.

Observations in the counting chamber should not be limited to merely tallying the numbers of red blood cells. Examine the relative size and shape of the erythrocytes. Normally there is uniformity of size and shape of the cells. In pathologic blood, lack of uniformity is often apparent.

As soon as the count has been made, clean the hemacytometer and coverslip in cold running water and dry with a clean, lint free cloth or lens paper. Do not allow preparations in the counting chamber to dry and do not perform counts on specimens which are drying. Clean the pipettes immediately, or if that is impossible, empty them and fill at once with distilled water until such time as they can be cleaned.

Numerical Counts of Leukocytes

Normal values of leukocytes are:

Adults: 4,500 to 11,500, average 7,400 per cu. mm.

Equipment and Reagents.—

The blood specimen may be capillary or venous oxalated blood.

White Blood Cell Diluting Pipette.—There are two types of pipettes, the classical Thoma and the Trenner. The Thoma pipette, Fig. 152, has 10 divisions on the capillary end with 0.5 and 1.0 prominently scored. Beyond the bulb is another mark, 11, which indicates the level to which diluting fluid is drawn. If blood is drawn to the 0.5 mark, the resultant dilution is 1:20 (1 volume of the total 11 volumes remains in the stem and is blown out before the counting chamber is filled, leaving a total of 10 volumes of diluted blood). For routine leukocyte counting, a dilution of 1:20 is used.

If marked leukopenia is present, a lesser dilution may be obtained by drawing blood to the 1.0 mark, a dilution of 1:10. Various dilutions to suit particular conditions may be obtained by drawing blood to any of the other divisions on the capillary pipette. The Trenner or "automatic" pipette permits of only one dilution, 1:20.

Diluting Fluid.—**1% or 2% Acetic Acid.—**

Use 1 c.c. (or 2 c.c.) of glacial acetic acid for each 100 c.c. of counting solution. Filter three times through the same filter paper. The solution must be made fresh at least once a week.

The addition of a few drops of methylene blue or brilliant cresyl blue to 100 c.c. of the solution will stain the leukocytes and aid in visualizing them under the microscope.

Filter again immediately before use.

N/10 Hydrochloric Acid.—

This diluent is used routinely when the Haden-Hausser hemoglobinometer is used. No dye should be added to this solution. Method of making normal solutions is given on page 24.

Hemacytometer.—

This is described on page 570.

The four corner squares, marked "W" in Fig. 153, are counted routinely. These are 1 sq. mm. each. One square covers a low-power, dry objective field with a 10× ocular (16 mm. objective).

The formula for determining factors in hemacytometry is given on page 570.

Hemacytometer Coverglass.—Use no substitute coverglasses.

Microscope.—**Technic.—**

Prepare the patient as directed on page 572 and make the puncture. Wipe off the first drop of blood.

Draw the blood to the mark 0.5 (for routine counting) in the capillary end of the pipette, and the counting solution to the mark 11. Use the same technic as described under red blood cell counting. Be sure to wipe the blood off the outside of the pipette before immersing the pipette in the watch glass containing the counting solution.

Shake as for a red count, allow several drops to leave the pipette, and immediately fill both sides of the counting chamber.

Focus under low-power, dry magnification, preferably with the condenser removed or lowered. Adjust the light so that the leukocytes appear as slightly iridescent round bodies with a very definite outline. If there is doubt as to whether an object is a leukocyte or an artefact, look at it under high magnification. Leukocytes have definite cell outlines and well-defined nuclear structure. Platelets appear as very small refractive bodies about one-fifth to one-seventh the size of the leukocytes, with no nuclei, and slight Brownian movement. Erythrocytes should not be present because the diluting fluid should hemolyze them. Occasionally ghost outlines of red cells may be seen.

For routine counting, enumerate the cells in the 4 corner "W" squares, each divided into 16 smaller squares for convenience in counting. Count first the upper left square of 16 small squares, then the upper right, the lower right, and last the lower left. The enumeration should follow the same pattern described under erythrocyte counting. Include in the count those leukocytes which are half in and half out of the upper and left-hand lines, but not those half in and half out of the lower and right-hand lines. Remember that the boundary line is the center line of the triple ruling.

Count two such drops, one in each side of the counting chamber.

Record the counts, and obtain the sum. Or use a mechanical counter.

Calculation.—**For Routine Leukocyte Counts:**

Multiply the count for 1 drop (total of 4 "W" squares) by 50.

194 cells counted in the first drop.

198 cells counted in the second drop.

2) 392 cells

196 leukocytes counted in 4 "W" squares.

Dilution of the blood 1:20. Area counted 4 sq. mm.

$V = \frac{1}{20} \times \frac{1}{10} \times 4 = \frac{1}{50}$ cu. mm.

$C = 196 \times \frac{1}{\frac{1}{50}} = 196 \times 50 = 9,800$ leukocytes per cu. mm.

Comment: *In routine leukocyte counting only, where 4 "W" squares are counted, the mathematics may be simplified by dividing the number of cells counted in a total of 4 "W" squares by 2 and adding two 0's. An average of the two counts must be made.*

Example for Leukopenic Blood:

98 leukocytes counted in 8 "W" squares.

Dilution of blood 1:10 (blood drawn to mark 1 instead of 0.5).

Area counted 8 sq. mm.

$$V = \frac{1}{10} \times \frac{1}{10} \times 8 = \frac{8}{100} \text{ cu. mm.}$$

$$C = 98 \times \frac{1}{\frac{8}{100}} = \frac{9,800}{8} = 1,225 \text{ leukocytes per cu. mm.}$$

Example for Blood With Hyperleukocytosis:

600 cells counted in 4 "W" squares (4 sq. mm.).

Dilution of blood 1:33 (blood drawn to 0.3 mark in pipette).

$$V = \frac{1}{33} \times \frac{1}{10} \times 4 \text{ cu. mm.} = \frac{4}{330} \text{ cu. mm.}$$

$$C = 600 \times \frac{1}{\frac{4}{330}} = \frac{600 \times 330}{4} = 49,500 \text{ leukocytes per cu. mm.}$$

Comment on Hyperleukocytosis:

In instances where leukocyte counts exceed 50,000 per cu. mm., dilutions may be made in the red cell diluting pipette, using white counting solution. If blood is drawn to the 1.0 mark in the red pipette, the dilution is 1:100.

If a specimen is taken routinely in the white counting pipette and it is found that the count is too high to enumerate accurately, and the patient is not available for another dilution to be made, count the cells under high magnification in the center square (the one divided into 400 small squares), counting all the cells in the entire 25 squares, or 1 sq. mm. Do not take an average. The area counted is 1 sq. mm., the dilution of blood is 1:20, and the depth of the counting chamber is $\frac{1}{10}$ mm.

Multiply the sum of the cells by 200.

During the counting process, observe for equality in distribution of the cells. If clumps are present, the distribution is unsatisfactory and an entirely new dilution of blood is required.

As soon as tallying has been completed, clean the hemacytometer and coverslip in cold running water and dry with a clean lint-free cloth or lens paper. Do not allow specimens in the counting chamber to dry and do not perform counts on preparations which are drying. Clean the counting pipettes immediately, or if that is not possible, empty them, and fill with distilled water until such time as they can be cleaned.

Historical.—

The procedure of counting the total number of white cells in the blood seems to have been initiated by Malassez in 1872.¹ The method of studying the white and red cells was worked out further by Gowers in 1877, Thoma in 1882, who used the red pipette and modified it for the leukocytes, thus giving us the pipette so universally used ever since; Alferow in 1884, who distinguished white cells by raising the tube to lengthen focal distance; Toisson in 1885, who used a fluid permitting the counting of white cells in the same pipette with reds. This ideal was pursued by Schüffner further in 1911 and Türk in 1912. Zappert in 1892 worked out the first satisfactory ruling for the counting chamber. Rieder in 1892, Elzholtz in 1894, Friedlander in 1897, Durham in 1897, Einhorn and Laporte in 1902 (white count being made directly from film, by noting the ratio of white cells to red cells and counting only the red cells in chamber), Türk in 1902, Breuer in 1902, Joffroy and Mercier in 1902, Engel in 1902 (differentiation of white cells by refraction of light, most like Alferow), Burkner in 1905 and in 1907, Ellermann and Erlandsen in 1909, Dunger in 1910, Galambos in 1910, Hill in 1912, and Roderdanz in 1913 further worked out the details of counting white cells.

Method of Making Numerical Counts of Leukocytes in Blood Showing Large Numbers of Nucleated Red Cells

There are some conditions accompanied by an influx of nucleated red cells into the blood stream. When leukocyte counts are made of such bloods, it is often difficult, and sometimes impossible, to differentiate under low magnification between the leukocytes and the nuclei of these erythrocytic cells, which are not dissolved by acetic acid. Under low magnification these nuclei,

¹Malassez, L.: *Comptes rendus et memoires de la Soc. de biol.* 19 Oct. 1872, 5 Serie, 4, (24) 213; and 23 Nov. 1872, S. 5, Vol. 4, p. 236.

and nuclei plainly visible under high magnification. Many of the leukocytes will show the typical lobulated appearance of the granulocytes, but some will have round or oval nuclei. The nuclei of the erythrocytes will be spherical, refractive, and devoid of cytoplasm.

Count all the leukocytes in the center square usually used for counting erythrocytes. Count under high magnification without the Abbé condenser. Count all 25 of these high-power fields, making a total of 1 sq. mm. counted. Do not take the average. Count two or more such fields and take the average of the 2 or 3 counts.

Calculation.—

The number of cells in the 1 sq. mm. fields (25 red fields) \times 200 gives the number of leukocytes in 1 cu. mm. blood, provided the count was taken in the usual manner.

Example.—

114 leukocytes counted; $114 \times 200 = 22,800$ leukocytes in 1 cu. mm. blood.

Cleaning the Pipette

Eject the contents of the pipette immediately after using. Never allow the blood to remain in the pipette, as this might cause permanent blockage. Use a suction pump to clean the pipette, drawing the following various solutions through it.

Wash thoroughly with distilled water until all traces of blood have been removed. Next, draw alcohol through the pipette to remove the water. Next, draw ether through the pipette to remove the alcohol, and to dry. Continue the use of suction until the pipette is thoroughly dry, as evidenced by the jumping of the glass bead in the bulb. If the glass bead adheres to the side of the bulb, the pipette still contains moisture, which must be removed with alcohol and ether.

Ammonia water, potassium hydroxide, or N/10 hydrochloric acid may be used in cleaning the pipette, provided all traces of the chemical employed are removed before using the pipette again.

Some laboratories prefer to clean the pipettes with distilled water, followed by drawing acetone through the pipettes, then an air current to dry them.

CAUTION

If the pipettes are set aside after the dilutions are made, the contents must be thoroughly mixed before filling the counting chamber, as the cells will tend to settle at the bottom of the bulb, and subsequent counts without mixing will be inaccurate.

In making counts, count all cells within the ruled area, and, in addition, all cells which lie along the ruled lines at the top and left side of the field. Omit all cells lying on the bottom and right sides, unless they are definitely within the ruled area. Remember that the center line of the triple ruling is the border of the field in some counting chambers. One must study the rulings to distinguish between guide lines and boundaries of fields.

Precautions to Be Observed in Making Red and White Counts and Blood Films

1. Always remove the first drop or two of blood which oozes out after the skin puncture. Always remove the blood which is left between taking of the various specimens, such as red and white counts, films, etc.
2. Never, under any circumstances, squeeze to obtain blood.
3. Hold the pipettes either vertically or up at a 45 degree angle; never allow them to hang down from the puncture.
4. If blood clots occur in the red and white counts, in spreads, thick drop, or hemoglobin test, repeat the preparations to avoid inaccuracy.
5. Be sure to draw the blood for red and white counts and hemoglobin exactly to the mark on the pipette—never above nor below.
6. Be sure that no air bubbles enter the pipette during the diluting process.

7. Always be sure there is no blood on the outside of the pipette before drawing up the counting solution.

8. Shake the pipettes for several minutes before filling the counting chamber, but do not shake them too hard.

9. Eject several drops of diluted blood from the pipette before filling the counting chamber. If more than one drop of the dilution is counted, the pipette must be shaken before filling the counting chamber the second time and several drops of the dilution must be ejected.

10. Never overfill nor underfill the counting chamber. If air bubbles are present, clean the counting chamber and refill. If the counting chamber is imperfectly overfilled, clean the counting chamber and refill with a fresh drop.

11. It is well to take the specimens for the complete blood picture in the following order: films, white count, red count, thick drop, hemoglobin.

12. Under no circumstances allow the pipettes for red and white counts and hemoglobin, or the slides for films and thick drops, to touch the skin.

13. When diluting the red and white counts, be sure that no blood runs back out of the pipettes while drawing up the diluting fluid.

14. Keep blood films and thick drops out of dust and away from flies and other insects.

15. Always make the counts as soon after taking the specimen as possible.

16. Use fresh counting solution for each count. Filter enough solution into a watch glass or small tube to be used for diluting one count. Do not dip the pipette into the stock bottle of counting solution.

17. Never use oxalated or heparinized blood for making blood films.

HEMOGLOBIN DETERMINATIONS

Hemoglobin is an iron-bearing protein which is the red coloring matter of the blood. Discussion of hemoglobin is found on pages 548 and 734.

Normal Values of Hemoglobin:

Adult males: 14.0 to 18.0 gm. per 100 c.c. blood, average 15.8. Adult females: 11.5 to 16.0 gm. per 100 c.c. blood, average 13.9.

Methods in Hemoglobinometry

There are four basic methods of measuring hemoglobin content of the blood:

A. Colorimetric.

1. Hemoglobin examined directly as oxyhemoglobin by the methods of Dare, Tallqvist, Spencer instrument, and the photoelectric method.

2. Hemoglobin converted to acid hematin by the methods of Sahli, Newcomer, Haden-Hausser, LaMotte, and the photoelectric method.

3. Hemoglobin converted to alkaline hematin in photoelectric methods.

4. Hemoglobin converted to CO-hemoglobin by the method of Haldane.

5. Hemoglobin converted to cyanmethemoglobin.

B. Measurement of Specific Gravity of Whole Blood and Serum or Plasma.—The copper sulphate method.

C. Gasometric Measurement of Oxygen Capacity of Blood.—The method using the Van Slyke apparatus.

D. Measurement of the Iron Content of Blood.

A. Colorimetric Methods

Dare Method

Normal Values: Instrument is standardized so that 16 gm. per 100 c.c. of blood is the equivalent of 100 per cent hemoglobin.

Principle.—

The Dare hemoglobinometer is based on the principle that the color of a thin film of undiluted blood, illuminated by a standard light source, may be compared with a graduated, nonfading color scale built into the apparatus. Models are available in both battery or electrically operated light sources. The matching fields are supplied in two types, regular or juxtaposition fields.



Fig. 154.—Dare hemoglobinometer with the gram scale. (Courtesy Rieker Instrument Co., Philadelphia.)

Technic.—

Prepare the instrument by placing the two pieces of glass (one frosted and one plain) together and inserting them into the small metal holder.

Make a puncture in the usual manner, discarding the first drop of blood. Allow the blood to run into the space between the two pieces of glass; then place the specimen in the instrument so that the white glass will be toward the source of light.

Look through the telescope, holding the hemoglobinometer in a position which allows the light to penetrate the blood specimen.

Revolve the standard by turning the knurled knob at the top of the instrument, until the color of the unknown exactly matches that of the standard.

Read from the graduation marks at the side of the instrument the percentage or grams of hemoglobin.

To convert to grams of hemoglobin per 100 c.c. blood, multiply the percentage of hemoglobin by 16.00,¹ or convert to grams by reading directly off the scale.

Example.—

Percentage of hemoglobin is 95.

$$0.95 \times 16.00 = 15.20.$$

After use, the instrument should be cleaned and dried immediately.

¹See page 590.

Spencer Hemoglobinometer*

The Spencer hemoglobinometer is a battery or electric current-operated instrument, pocket size, and very convenient for determining hemoglobin concentration. It utilizes light in the visible green spectrum. In addition to the hemoglobinometer, hemolysis applicators are needed.

Technic.—

Place a large drop of blood, from a skin puncture, directly onto the open glass chamber of the apparatus. Hemolyze it completely with a hemolysis applicator until blood change is apparent. This takes approximately 45 seconds. Close the chamber by sliding it under the cover glass, and insert into the instrument. The hemolyzed blood must entirely fill the chamber; there must be no air spaces.

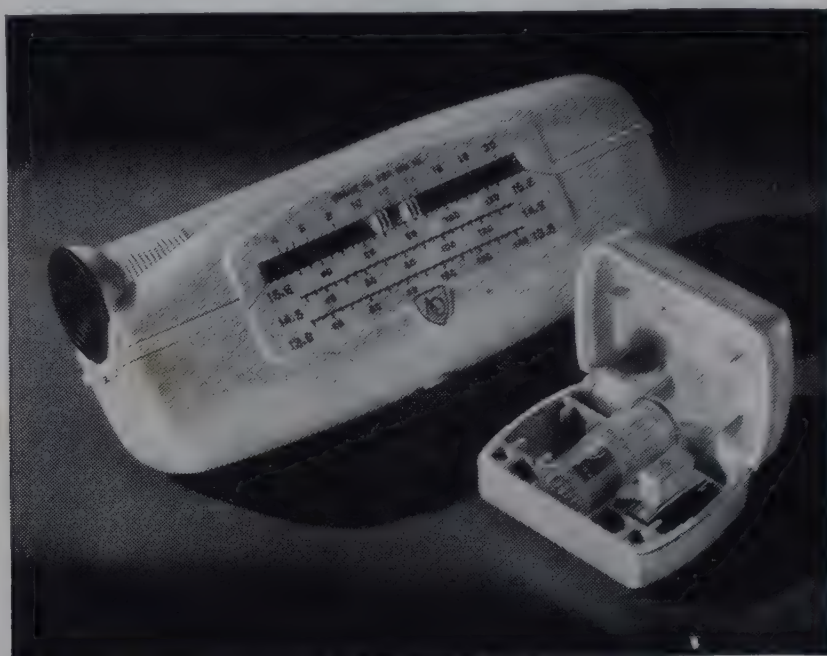


Fig. 155.—The Spencer hemoglobinometer. (American Optical Company, Scientific Instrument Division.)

Press the illuminating switch, and observe through the eyepiece. There will be seen two halves of a circular field, one of which is stationary and represents the standard and the other which is movable and represents the unknown. By moving the lever on the side of the instrument, the fields can be brought to match each other. It is well to take several readings until two consecutive readings are alike.

Read the hemoglobin concentration directly from the graduated scales on the side of the apparatus. There are four scales on the instrument: grams per 100 c.c., per cent scale based on a standard of 15.6 gm. per 100 c.c. blood, per cent scale on a basis of 14.5 gm., and per cent on a standard of 13.8 gm.

The inclusion of four scale readings on this apparatus is magnificent testimony to the widespread confusion as to what level of hemoglobin concentration is "normal."

Sahli Method

Normal Values: Sahli instruments have been on the market calibrated so that the "normal" values were: 100 per cent equivalent to 13.8, 14.0, 14.5, 17.0, 17.2, and 17.3 gm. of hemoglobin per 100 c.c. of blood.

The Sahli hemoglobinometer consists of a set of glass rods colored amber, and housed in a casing with a central aperture. Into this center fits a graduated tube with a scale reading either in grams of hemoglobin per 100 c.c. of blood or percentage of hemoglobin. The Sahli pipette has a capacity of 20 cu. mm. (0.020 c.c.). N/10 hydrochloric acid is used to convert to acid hematin.

*The Spencer hemoglobinometer is manufactured by the American Optical Co., Scientific Instrument Division, Buffalo, N. Y.

Technic.—

Prepare the instrument by placing N/10 hydrochloric acid in the graduated tube until the meniscus stands at the mark 10 on the percentage scale.

Draw blood into the Sahli pipette to the mark 20. Wipe the blood off the outside of the pipette.

Eject the blood into the hydrochloric acid solution and mix by drawing in and out of the pipette several times. Blow through the pipette while removing it from the tube.

Shake the tube and allow to stand a minimum of 1 minute and a maximum of 10 minutes.

Add distilled water a drop at a time, mixing after the addition of each drop, until the color in the tube corresponds exactly to the color of the standard. Do not go beyond the point where exact color matching has occurred. If this happens, repeat the entire procedure.

Read in north daylight and turn the graduations to the side so as to read through clear glass.



Fig. 156.—Sahli hemoglobinometer. (Courtesy E. H. Sargent & Co.)

Read from the scale the percentage of hemoglobin. To convert to grams per 100 c.c. of blood, multiply the percentage figure by the gram standard on the tube.

Comment: This acid hematin method is simple. The apparatus is relatively inexpensive. The variety of standards is confusing and the visual matching is subject to considerable individual variation.

Newcomer Method

Normal Values: The Newcomer standard is 16.92 gm. of hemoglobin per 100 c.c. of blood.

The equipment consists of a standard Newcomer colored disk. Note the thickness inscribed on the disk. A Klett or Duboseq type colorimeter and a Sahli hemoglobin pipette are used.

Place the standard Newcomer disk in the Duboseq or Klett colorimeter preparatory to the reading of the hemoglobin test.

Place 5 c.c. N/10 hydrochloric acid in a colorimeter cup.

Draw the blood to the 20 mark in a Sahli pipette.

Eject the blood into the hydrochloric acid, and mix by drawing in and out of the pipette several times. Note the time at which the blood enters the acid solution.

Mix the contents of the cup well, and allow to stand 10 minutes.

Have the cup below the standard disk filled with distilled water. Be sure the plunger enters the distilled water.

Match the colors in the colorimeter, and obtain the reading. Calculate the grams of hemoglobin per 100 c.c. of blood by dividing the reading in Table 48 by the reading on the colorimeter.

TABLE 48

MINUTES SINCE DILUTION	THICKNESS OF THE COLORED GLASS IN MILLIMETERS										
	0.95	0.96	0.97	0.98	0.99	1.00	1.01	1.02	1.03	1.04	1.05
10	93.4	95.4	96.4	97.4	98.4	99.4	100.4	101.4	102.3	103.4	104.4
15	93.1	94.1	95.0	96.0	97.0	98.0	99.0	100.0	101.0	102.0	103.0
20	92.5	93.5	94.5	95.4	96.4	97.4	98.4	99.4	100.4	101.3	102.3
30	91.8	92.8	93.8	94.8	95.7	96.7	97.7	98.6	99.6	100.5	101.5
40	91.6	92.5	93.5	94.5	95.4	96.4	97.4	98.3	99.3	100.3	101.2
Final	90.6	91.6	92.5	93.5	94.4	95.4	96.4	97.3	98.3	99.2	100.2

Table 48 takes into account the time which has elapsed before the reading was made and the thickness of the glass standard. The exact thickness of the glass disk is engraved on it.

Example.—

Ten minutes elapsed before the reading was made.
Thickness of glass disk was 1.00 mm.
Reading on the colorimeter was 6.7.
 $99.4 \div 6.7 = 14.83$.

Haden-Hausser Method¹

Normal Values: The Haden-Hausser standard is 16.7 gm. of hemoglobin per 100 c.c. of blood, which is equivalent to 100 per cent.

The Haden-Hausser hemoglobinometer is manufactured in two models, the clinical and the laboratory. The basic principles and general design of the two instruments are essentially the same. The laboratory model is larger and features an electrically controlled light source. A miniature comparator scale, graded from 7.5 to 19.0 gm. of hemoglobin, is built into a well in the apparatus. This is overlaid by a removable, fitted, glass cover-slip, which, when in position, directs the flow of the blood sample into the chamber.

A white blood counting pipette is used.

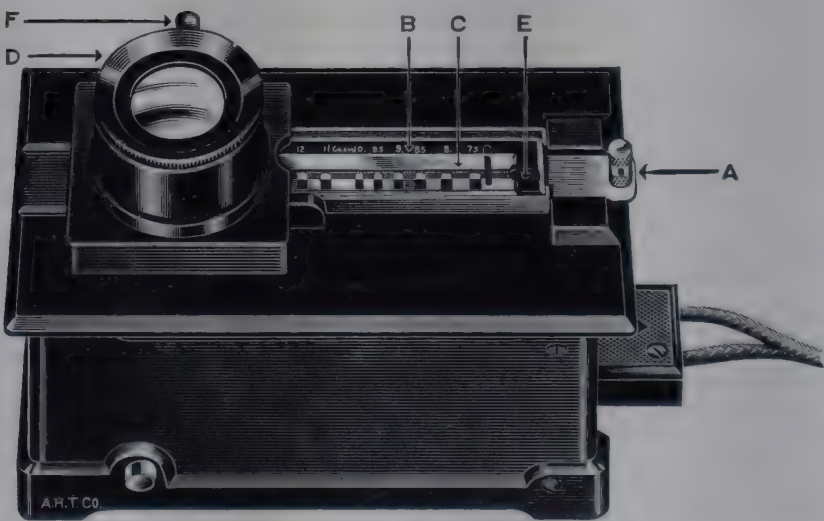


Fig. 157.—Haden hemoglobinometer. (Courtesy Arthur H. Thomas Co.)

Technic.—Dilute the blood 1:20 or 1:10 in a white blood cell counting pipette with N/10 hydrochloric acid.

Allow to stand at least thirty minutes to complete conversion of the hemoglobin into acid hematin.

Run the diluted blood into the dilution channel of the comparator.

Move the comparator until a matching of the blood specimen and a colored segment of the comparator is found. Read values directly from the instrument.

¹Haden, R. L.: J. Lab. & Clin. Med. 16: 68, 1930.

Comment: An advantage of this method is that since the dilution of the blood is made in a white blood cell counting pipette, both the leukocyte count and the hemoglobin estimation can be carried out with the same diluted blood specimen.

Photoelectric Hemoglobin Determinations Based on the Measurement of Oxyhemoglobin

Normal Values: Normal values vary with the method and standardization of the photoelectric colorimeter. Values should preferably be expressed in grams of hemoglobin per 100 c.c. of blood.

Technic.—The methods of determining hemoglobin by photoelectric colorimetry are given on pages 280 to 284.

In the original method of Sheard and Sanford, which was the first photoelectric hemoglobin determination, 0.1% sodium carbonate was used as a diluent. The color of the resultant solution tends to fade within 3 to 4 hours, so that reading of the test must be made within this time limit. It has been found that 0.04% ammonium hydroxide will keep oxyhemoglobin solutions stable for as long as 4 days.¹ Hypotonic saline solutions have been generally abandoned as diluting fluids.

Photoelectric Determination of Hemoglobin Based on the Measurement of Acid Hematin

Normal Values: Normal values vary with the technic and the standardization of the photoelectric colorimeter. The Fisher Electro-Hemometer (Fig. 158) uses a normal standard of 15.6 gm. of hemoglobin per 100 c.c.



Fig. 158.—The Fisher Electro-Hemometer. (Fisher Scientific Co., Pittsburgh, Pa.)

The Fisher apparatus has an ammeter reading scale limited to percentage and grams per cent of hemoglobin. Any photoelectric colorimeter may be calibrated for this method. N/10 hydrochloric acid is used as the diluent. It takes about 30 minutes for full development of the brown color; this time should be the minimum allotted for reading unknown samples.

Technic.—

Use either venous or finger blood.

Draw the blood into the hemo-pipette exactly to the graduation mark below the bulb and wipe the tip of the pipette clean.

¹Bell, G. H., Chambers, J. W., and Waddell, M. B. R.: *Biochem. J.* 39: 60, 1945.

Draw N/10 hydrochloric acid into the pipette to exactly the graduation mark above the bulb.

Allow the mixture to remain in the pipette for 30 minutes, or transfer it to the Fisher photometric cell and allow it to remain for 30 minutes.

Be sure that the pointer reads on the line marked "A." Adjust by rotating the knob on the top of the instrument at the point marked "Adjust Zero."

Turn the switch to "On" and allow the instrument to remain for 1 to 2 minutes. Insert the standardizing cell, containing distilled water, through the hole just in front of the reading scale. Turn the knob on the front panel and adjust the pointer to the line "B."

Remove the water-filled cell and replace with cell containing the blood specimen.

The pointer now indicates the hemoglobin concentration of the blood specimen both in grams of hemoglobin per 100 c.c. of blood and in per cent of normal.

Comments: This method has generally been discarded in favor of methods using oxyhemoglobin. Basically the procedure is the same as that used in the Sheard and Sanford method for oxyhemoglobin (pages 280 and 283) except that N/10 hydrochloric acid is used.

Methods in Which Hemoglobin Is Converted to Alkaline Hematin

These are photoelectric methods which basically measure dilutions of blood treated with alkali for conversion of hemoglobin to alkaline hematin. These dilutions are read photoelectrically, usually with a filter at 540 $m\mu$. The instrument is calibrated against known solutions of hemoglobin. Graphs are prepared from the readings from known solutions and unknown determinations made from the graphs. Determination of hemoglobin based on alkaline hematin has not gained popularity and is rarely used.

Methods in Which Hemoglobin Is Converted to Carbon Monoxide Hemoglobin (Carbon Monoxyhemoglobin)

Normal Values: In the original Haldane methods the standard was 13.8 grams of hemoglobin per 100 c.c. of blood.

The colorimeter may be the Duboscq type of visual colorimeter or a photoelectric instrument. In the following method, a photoelectric apparatus is used, with a green filter (530 to 540 $m\mu$).

Reagents.—

0.4% Ammonium Hydroxide Solution.—

Dilute 4 c.c. of ammonium hydroxide to 1,000 c.c. with distilled water, in a liter volumetric flask.

Carbon Monoxide.—

CO may be obtained by running a stream of illuminating gas through the specimen, or by using commercially available tanks of carbon monoxide.

Methods in Which Hemoglobin Is Converted to Cyanmethemoglobin

Normal Values:

Adult males: 14.0 to 18.0 gm. per 100 c.c. blood; average 15.8 gm. %.

Adult females: 11.5 to 16.0 gm. per 100 c.c. blood; average 14.9 gm. %.

Reagent.—

Drabkin's Solution.¹—

Sodium bicarbonate	1.0 gm.
Potassium cyanide	52.0 mg.
Potassium ferricyanide	198.0 mg.
Distilled water	1,000.0 c.c.

Technic.—

Use a calibrated Sahli pipette to deliver 0.02 c.c. of capillary or venous oxalated blood to 5.0 c.c. of Drabkin's solution in a test tube. Rinse the blood out of the pipette

¹Drabkin, D. L.: Am. J. M. Sc. 215: 110, 1948; *ibid.* 217: 710, 1949.

repeatedly in the Drabkin's solution. Mix by swirling tube. Allow mixture to stand 10 minutes. If mixture is turbid, add a pinch of saponin to completely hemolyze the red cells. Read in a photoelectric colorimeter with a green filter in the range of 530 to 540 m μ . Stadie² has outlined a procedure for cyanmethemoglobin for visual colorimetry.

Comments: The cyanmethemoglobin method of hemoglobinometry has been recommended by a panel formed under the aegis of the National Research Council with a number of sponsoring groups.³ This panel has planned an extensive field trial of the method in selected laboratories throughout the United States. For this purpose, three standard certified solutions of cyanmethemoglobin, corresponding to 1:250 dilutions of blood containing 5, 10, and 15 gm. of hemoglobin per 100 c.c. of blood, are being prepared for distribution to laboratories. These standard solutions are to be used for calibration of the photoelectric apparatus. Readings on the photoelectric apparatus for each of the three solutions are transcribed on semilogarithmic paper and a line is drawn through these three points. This chart provides a correlation of photoelectric readings and concentration of hemoglobin in grams per 100 c.c. of blood. Since these standards are stable for years⁴ if kept sealed and refrigerated, they may be used as often as desirable for calibration. Such ready availability of standardization should serve as an excellent check on photoelectric apparatus.

The use of a solution of cyanide in routine laboratory work poses the question of safety. Crosby and associates⁴ take cognizance of this danger and recommend the following precautions:

- "1. Pipettes should be filled with a suction bulb.
- "2. Blood should be mixed with the cyanide solution by swirling.
- "3. If small amounts of cyanide compounds are accidentally spilled on the laboratory bench or on the floor during the weighing procedure, the dry powder should be wiped up with a damp cloth and discarded in a suitable container.
- "4. Cyanide salts should be stored in a locked cupboard."

However, Drabkin's solution contains an amount of cyanide which is far below a lethal dose,* which has been estimated to be approximately 4 to 6 liters of the solution.

Although the panel previously mentioned recommends the adoption of the cyanmethemoglobin method, its members do not preclude the use of the cyanmethemoglobin standards for standardizing other methods of hemoglobin determinations. The results of Crosby and associates⁴ indicate that when the method of calibration and of hemoglobinometry were **both** based on cyanmethemoglobin, the correspondence was within $\pm 2.1\%$. With other combinations of calibration and method, results were as far off as 12.7% of the true value of the test solution.

B. Methods in Which Hemoglobin Determinations Are Based on the Specific Gravity of Whole Blood and Serum or Plasma

This method, frequently referred to as the "copper sulphate" method, is a useful rough guide in estimating hemoglobin concentrations and total proteins simultaneously.

*52 mg. of KCN per liter.

²Stadie, W. C.: *J. Biol. Chem.* 41: 237, 1920.

³Cannan, R. K.: *Am. J. Clin. Path.* 25: 376, 1955.

⁴Crosby, W. H., Munn, J. L., and Furth, F. W.: *U.S. Armed Forces M. J.*, 5: 693, 1954.

As a screening process it is of inestimable value, particularly where large numbers of determinations must be made. The method was developed during World War II and was popularized in the Armed Forces of the United States. It is not recommended as a routine clinical procedure. The method is described on page 284.

C. Methods of Hemoglobin Determinations Based on Gasometric Analysis

These determinations are performed in the Van Slyke-Neill apparatus. The methods originally were based on the oxygen capacity of blood. Various refinements, including the determination of carbon monoxide of the blood, have been added to the original method.¹ The method has a high degree of accuracy, but the procedure is too cumbersome to lend itself to routine testing.

Even with this highly refined method, there remain some questions concerning normal values of human hemoglobin. In the past, it was assumed that the constant for oxygen combining with each gram of hemoglobin (Hüfner's factor) was 1.34 volumes per cent. These data were based on observations in lower animals, in which it was found that the constant is 1.36 volumes per cent.² Using the old factor, 20.9 volumes per cent oxygen capacity of blood, the hemoglobin determination follows:

Grams of hemoglobin per 100 c.c. of blood = $\frac{20.9}{1.34} = 15.58$. With the factor, 1.36 volumes per cent, this same oxygen capacity gives the following hemoglobin determination:

$$\text{Grams hemoglobin per 100 c.c. of blood} = \frac{20.9}{1.36} = 15.37$$

This material is presented chiefly to emphasize the fact that, even with methods of extreme precision, universal agreement on normal values for hemoglobin has not been reached. Part of this disagreement stems from the complex nature of hemoglobin, but the gaps in our knowledge are being closed by more extensive study, including improvements in technical aspects.

Gasometric analysis lends itself well to calibration of clinical hemoglobinometers.

D. Methods of Hemoglobin Determinations Based on the Measurement of Iron Content of the Blood

Basically, the many modifications for determining iron content of blood depend upon liberation of the four iron molecules attached to the one globin molecule in hemoglobin. The Wong method (pages 281 through 283) is a typical technic. The subject of iron determination in blood has been very thoroughly covered in a symposium on hemoglobinometry.³ The iron method is too cumbersome for routine clinical work but its accuracy ($\pm 1\%$) has made it a popular method for the calibration of clinical methods.

Critique of Hemoglobinometry

Determination of hemoglobin is perhaps one of the most common laboratory procedures in a hematologic study of patients, and yet there exists more inaccuracy in this determination than in many other tests. The subject was of sufficient interest to warrant its presentation before the American Society of Clinical Pathologists and the College of American Pathologists in 1950. As a result, a most comprehensive monograph on clinical hemo-

¹Van Slyke, D. D., Hiller, A., Weisiger, J. R., and Cruz, W. O.: *J. Biol. Chem.* **166**: 121, 1946.

²Bernhart, F. W., and Skeggs, L.: *J. Biol. Chem.* **147**: 19, 1943.

³Sunderman, F. W., MacFate, R. P., MacFayden, D. A., Stavenson, G. F., and Copeland, B. E.: *Am. J. Clin. Path.* **23**: 519, 1953.

globinometry was published.¹ These authors state that "recent surveys have revealed wide discrepancies in hemoglobin measurements in different clinical laboratories. These are in part due to errors in measurements, differences in methodology, the uncertainties encountered with the continuance of obsolete color methods that use artificial standards, and, perhaps most important, because of a lack of an accepted standard of reference for hemoglobinometry." Belk and Sunderman² sent two samples of hemoglobin to 92 clinical laboratories. These samples contained 9.8 and 15.1 grams of hemoglobin per 100 c.c., respectively. Surprising as it may seem, the sample containing the 9.8 grams per 100 c.c. was reported with values as high as 15.5 grams and as low as 5 grams. Only 14 of the 92 laboratories analyzing this specimen were within the accepted range for the blood sample. The discrepancies with the second sample were almost as striking as with this first sample. Such discrepancies are obviously related to technical problems such as errors in technique, poorly standardized equipment, and equipment poorly adapted to accurate analysis.

Karr and Clark³ call attention to inaccurately calibrated pipettes, both for blood sampling and for measuring diluents, as a source of error. They also include careless technique as an important factor in poor results. For accurate hemoglobin determination, all equipment used must be calibrated and standardized. If it is impossible to calibrate instruments because of lack of properly trained personnel, the work can be done by experts either in the vicinity or by supply houses which furnish such equipment. Once a photoelectric colorimeter has been calibrated, it should be checked from time to time for accuracy. Refer to the section on photoelectric colorimetry for further information (pages 208 to 221).

Regarding methodology, today we are solving one of the problems that has plagued us in hemoglobinometry for the past 75 years, by the use of photoelectric colorimetry. Obviously such precision instruments have a considerable advantage over all methods utilizing visual colorimetry. However, the use of a precision instrument does not guarantee precise results unless all technical aspects in the use of the instruments are properly understood and carried out.

It would be exceedingly helpful if there could be some universal agreement as to the normal values for hemoglobin. Here in the United States, it is generally agreed that the average normal hemoglobin concentration for males is 15.8 gm. per 100 c.c. of blood, and for females, 13.9.⁴ Since these figures represent accumulations of great numbers of observations, it is suggested that this be used as a standard for normals in the United States. From a clinical standpoint, it is important to remember that there are ranges of hemoglobin values that are still within the normal range for both males and females. Such ranges, as given by Albritton, are, for adult males, 14.0 to 18.0 gm. per 100 c.c., and for adult females, 11.5 to 16.0 gm. per 100 c.c. Acceptance of these

¹Sunderman, F. W., MacFate, R. P., MacFayden, D. A., Stevenson, G. F., and Copeland, B. C.: *Am. J. Clin. Path.* 23: 519, 1953.

²Belk, W. P., and Sunderman, F. W.: *Am. J. Clin. Path.* 17: 853, 1947.

³Karr, W. G., and Clark, J. H.: *Am. J. Clin. Path.* 11: 127, 1941.

⁴Albritton, E. C.: *Handbook of Biological Data, Standard Values in Blood*, Wakefield, Mass., 1952, McGregor and Werner, Inc., p. 38.

ranges of normal values must not be construed as placing a seal of approval on poor technic which results in variations perhaps on the same sample of blood.

The time-honored tradition of reporting values in percentage is so well established that it has been difficult in many places to discard this method of reporting. The time has now come when reporting hemoglobin in percentages should be discontinued. A most casual perusal of the various "normals" for 100 per cent for the different instruments used in hemoglobinometry reveals great disparity between the number of grams of hemoglobin per 100 c.c. that equals 100 per cent. In Table 49 are figures indicating some of the values considered 100 per cent in various methods.

TABLE 49

INSTRUMENT	GM. HB. PER 100 C.C. BLOOD
Sahli	13.8, 14.0, 14.5, 17.0, 17.2, 17.3, etc.
Sahli-Leitz	15.0
Dare	13.75, 13.77, 13.8, 15.9, 16.9
Tallqvist	13.8, 15.6, 15.8, 16.25, 16.5
Newcomer	16.92
Haldane	13.8
Haden-Hausser	15.4, 15.6, 16.7
von Fleischl-Miescher	15.8
Van Slyke	16.9

An interesting exercise is the conversion of per cent of hemoglobin, using some of the above standards.

To convert from per cent of hemoglobin to grams of hemoglobin in 100 c.c. of blood, multiply the grams standard of the apparatus used by the per cent of hemoglobin as read on the hemoglobin scale.

Examples:

- (a) 90% hemoglobin, Sahli. The Sahli standard is 17.3 gm.

$$\begin{array}{r} 17.3 \\ \times 0.90 \\ \hline 15.57 \end{array}$$
15.57 gm. hemoglobin in 100 c.c. blood
- (b) If the Sahli standard is 13.8 gm. per 100 c.c., then 90% = 12.42 gm. hemoglobin.
- (c) If the Haden-Hausser apparatus with a standard of 16.7 gm. is used, 90% = 15.03 gm.

It should be readily apparent from these examples that reporting hemoglobin values in percentage gives false implications.

If one feels compelled to adhere to the time-honored tradition of reporting in percentage, this should be accompanied by the standard upon which the percentage of hemoglobin is based, and one should bear in mind the range for normal values. Remember that the normal values for females are below those of males, so that what would be 100 per cent of normal for the mean average of females would be less than 100 per cent for males.

To convert from grams of hemoglobin per 100 c.c. of blood to per cent of hemoglobin, divide the number of grams in the patient's blood by the grams standard of the apparatus and multiply by 100.

The ultimate choice for hemoglobin determination should be related to the purpose for which determinations are made, with appreciation for the lim-

its of accuracy of the method to be used. Generally speaking, the less expensive types of equipment with relatively simple technics might be chosen in situations where screening is all that is desired. The Sahli, Haden-Hausser, Dare, and Spencer instruments fit into this category. These methods have shown errors ranging from ± 5 to 40 per cent, but "with a close check on the standards, it may be possible with careful technic to hold the error to ± 5 to 10 per cent."¹

In clinical laboratories, hospitals, and even in private offices of physicians, where a fair amount of hematology is performed daily, the cost of a well-standardized photoelectric colorimeter is not so prohibitive as to preclude its use routinely. In modest-sized hospital laboratories and in physicians' offices, it is recommended that the photoelectric colorimeter be of such construction as to permit its use for multiple determinations as well as of hemoglobin. Purchase of a photoelectric colorimeter which has been standardized for hemoglobin determinations only and which has the convenience of reading the concentration of hemoglobin directly from the ammeter on the instrument is not economical in the smaller laboratory. In large laboratories, where several photoelectric colorimeters are available, and where the use of one could be limited to determinations of hemoglobin, perhaps there may be some justification for the purchase of an instrument calibrated for hemoglobin only. Regardless of which type of photoelectric instrument is purchased, attention is again called to the fact that a *paramount prerequisite of its use is accurate calibration*. The fact that a hemoglobin determination has been performed with a photoelectric apparatus is no guarantee that it is accurate. This precision is directly proportional to the accuracy of calibration of the instrument, as well as precision in the performance of the technic.

Our method of choice for the clinical laboratory using photoelectric colorimetry is the Sheard-Sanford technic or some modification of it in which oxyhemoglobin is measured. Accuracy in this method is somewhere in the range of ± 4 per cent. Sunderman and associates¹ state that "it is the considered opinion of members of this panel that for routine clinical use, the carboxyhemoglobin methods, the cyanmethemoglobin methods, and the oxyhemoglobin colorimetric methods (in the order named) are the most reliable and trustworthy." The use of poisonous cyanide reagents in the cyanmethemoglobin method is a major objection to this method. Our objection to the carbon monoxyhemoglobin methods is the introduction of a complicating element (a tank of carbon monoxide or a source of illuminating gas) that is hardly justified for routine clinical procedures. This choice of oxyhemoglobin method is made with a full realization that it does not measure hemoglobin derivatives such as carboxy-, met-, and sulf-hemoglobin.

The gasometric methods are obviously unsuited for clinical hemoglobinometry, requiring special equipment and being time-consuming. The precision of such methods favors their use in the calibration of photoelectric instruments. However, many prefer methods based on iron analyses (accuracy ± 1 per cent) for calibration. These methods are likewise too cumbersome for routine clinical work.

¹Sunderman, F. W., et al.: loc. cit.

In the calculation of erythrocyte constants (mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration), the hemoglobin determinations must be based on methods having a high degree of accuracy. The photoelectric methods previously discussed meet these requirements.

Determination of Abnormal Hemoglobins by Electrophoresis

Abnormal hemoglobins may be demonstrated in the Tiselius apparatus. This equipment is expensive and procedures are laborious. A substitute for this apparatus has been found in the form of paper electrophoresis outfits.

The basic principle of the method is the observation of the migration of a drop of protein (stained or unstained) on a piece of filter paper moistened with buffer solution in an electrophoresis "hookup."^{1, 2, 3, 4}

The various hemoglobins detected by electrophoresis are known as hemoglobins A (normal adult), F (fetal), C, D, E, G, H, I, and S (sickle cell). Descriptions of the significance of these varieties of hemoglobin may be found on pages 734 ff. The relative mobilities of these hemoglobins have the following order: A, F, E, S, D, C.

The apparatus is described on pages 221 ff. and in Volume II, Toxicology.

Alkali Denaturation Test for Fetal Hemoglobin (Hemoglobin F)⁵

Normal Values:

Newborn infants	55 to 85% hemoglobin F
Aged 12 to 18 months	0 to 10%
Aged 1½ to 5 years	0 to 3%
Over 5 years	0 to 1.8%

Material Required.—

2 to 4 c.c. Oxalated Venous Blood

N/12 Potassium Hydroxide.—

Dilute 1 part of N/4 potassium hydroxide solution with 2 parts of distilled water.

Keep the stock solution in a refrigerator in tightly stoppered paraffin-lined bottle.

Precipitating Reagent.—

Add 400 c.c. of distilled water

2 c.c. of concentrated hydrochloric acid (10N)

to 400 c.c. of saturated solution of ammonium sulphate.

Conical Graduated Centrifuge Tubes

Toluene, c.p.

Physiologic Saline (0.85%).—(p. 2033.)

Stopwatch

Photoelectric Colorimeter

¹Kunkel, H. G., and Tiselius, A.: J. Gen. Physiol. 35: 89, 1952.

²Slater, R. J., and Kunkle, H. G.: J. Lab. & Clin. Med. 41: 619, 1953.

³Smith, E. W., and Conley, C. L.: Bull. Johns Hopkins Hosp. 93: 94, 1953.

⁴Motulsky, A. G., Paul, M. H., and Durrum, E. L., Blood 11: 897, 1954.

⁵Submitted by Dr. A. I. Chernoff as modification of original technic of Singer, K., Chernoff, A. I., and Singer, L.: Blood 6: 413, 1951; *ibid.* 6: 429, 1951.

Technic.—

Place the blood in a 15 c.c. conical centrifuge tube.

Wash once with saline, centrifuge, and discard washings.

Add 1.5 volumes of distilled water
and 0.4 volume of toluene.

Stopper and shake vigorously for 2 to 3 minutes and allow to stand overnight in a refrigerator at about 4° to 10° C. If it is desirable to proceed immediately, then shake for 5 to 6 minutes and continue without refrigeration.

Centrifuge for 20 minutes at 2,000 to 5,000 r.p.m.

Aspirate and discard upper clear layer and middle layer of sediment.

Filter the hemoglobin solution remaining in the centrifuge tube. The concentration of this hemoglobin solution will be between 8 and 12 gm. per cent and need not be checked.

Transfer 1.6 c.c. of N/12 potassium hydroxide solution to a Kahn tube and keep in a 20° C. water bath for several minutes.

Add 0.1 c.c. of the test solution of hemoglobin to the precipitating reagent, rinsing the pipette 6 times and agitating the tube gently 10 to 20 times. As soon as the hemoglobin solution has been added, time with the stopwatch.

At the end of exactly 1 minute, add 3.4 c.c. of the precipitating reagent of ammonium sulphate.

Stopper, invert 3 to 4 times, and filter into a clean tube (*Solution A*).

Determine the hemoglobin concentration of Solution A in a photoelectric colorimeter.

Prepare a 1:200 dilution of original hemoglobin solution by adding 0.02 c.c. (with a Sahli pipette) to 4 c.c. of distilled water (*Solution B*.)

Determine the hemoglobin concentration of Solution B in a photoelectric colorimeter.

Calculations.—

$$\text{Per cent fetal hemoglobin} = \frac{\text{Hemoglobin concentration Sol. A} \times \frac{1}{4}}{\text{Hemoglobin concentration Sol. B}} \times 100.$$

The factor $\frac{1}{4}$ is used to compensate for differences in dilutions of solutions A and B, which are 1:50 and 1:200, respectively.

Comments: For routine clinical studies, visual observation only of solution A may suffice since concentrations of 2% or more of hemoglobin F will impart a brownish or red color to the filtrate. Concentrations below 2% hemoglobin F result in colorless solutions. Diagnostically, values above 2% are significant and therefore this qualitative determination alone may be all the procedure necessary.

Attention is directed at the high concentrations (55 to 85 per cent) of hemoglobin F in newborn infants and the existence of elevated concentrations of this hemoglobin component generally up to 2 years of age. Occasionally, children up to 5 years of age may show slightly increased values (up to 3 or 4 per cent). Allowance should be made for these ranges in the study of disease under these conditions.

Elevations of alkali-resistant hemoglobin F occur almost constantly in the hereditary hemolytic syndromes. In thalassemia, concentrations up to 90 per cent hemoglobin F have been found, whereas in sickle cell anemia the concentration may reach 25 per cent. In hereditary spherocytosis, levels up to 20 per cent hemoglobin F are not unusual. The levels reached in a particular patient do not necessarily distinguish these various syndromes from one another.

The finding of moderate increases of fetal hemoglobin is not necessarily diagnostic of familial or hereditary hemolytic disease. Values up to 5 or 10 per cent have been found in chronic leukemia, multiple myeloma, and pernicious anemia. In almost all other anemias, such as iron deficiency anemia and acquired hemolytic anemias, hemoglobin F concentrations are normal.

VOLUME PER CENT OF ERYTHROCYTES (CELL VOLUME PER CENT OR HEMATOCRIT)

Measurement of the volume of packed red blood cells by the hematocrit technic is the most accurate and simplest method of determining the presence or absence of anemia or polycythemia, and of measuring their degree. This has been shown repeatedly in many publications, among the best of which are papers by Rosemary Biggs, in England. In comparison, hemoglobin determination is less accurate, and red blood cell counting far less accurate.

Relative to these determinations, M. M. Wintrobe,* in a personal communication, stated, "It has been our practice for years to do just this and to reserve the determination of hemoglobin and red cell counts for those instances where one wishes to know what the average size and hemoglobin content of the red cells are. When this is needed, then the hemoglobin should be measured carefully and the red cell counts should be carried out meticulously. Even then, red cell counting carries a substantial margin of error. Certainly, as red cell counting is done in ordinary practice, it carries an error of anywhere from 20 to 50 or more per cent. I see many instances of erroneous blood cell counting and, strangely enough, most physicians are unaware of the large margin of error in this determination."

When one wishes to know simply whether or not there is anemia or polycythemia, it is much better to make a hematocrit examination than to test for hemoglobin or make a red count.

In preoperative and postoperative hemorrhage cases, hematocrit examinations are very useful in controlling blood replacement.

VOLUME OF PACKED ERYTHROCYTES

Hematocrit

Normal Values:

Adult male: Average - 47 volumes per cent, range - 40 to 54. Adult female: Average - 42 volumes per cent, range - 37 to 47.

(I) Macromethod for Hematocrit Determination, Wintrobe

Equipment.—

Wintrobe Hematocrit Tube.—This is a heavy cylindrical tube marked off on the exterior on the left-hand column from 0 to 10 cm., and on the right from 10 to 0, beginning at the top of the tube. Each main division represents 1 cm., and each small division 1 mm. To convert the reading to percentage, when the tube has been filled to the top, multiply the figure in centimeters by 10.

Blood is collected by venepuncture and placed in 3:2 oxalate mixture (page 567) and mixed thoroughly but gently.

Hematocrit-Filling Pipettes.—These are long glass capillary pipettes or preferably metal cannulae which fit inside the Wintrobe tube.

Centrifuge should be capable of a speed of 3,000 r.p.m. or 2,000 g.

Technic.—

Mix the oxalated venous blood thoroughly.

Draw up sufficient blood to fill a Wintrobe tube, slightly more than 1 c.c., in a dry filling pipette.

*Personal communication: M. M. Wintrobe, Professor and Head of the Department of Medicine of the University of Utah.

Insert the tip of the pipette into the bottom of a clean, dry Wintrobe hematocrit tube. By gentle pressure of the rubber bulb at the end of the pipette, fill the hematocrit tube, slowly pulling up on the pipette. Avoid introducing air bubbles. The tube should be filled to exactly the 0 on the left side of the tube. If the tube is filled beyond this point, remove enough blood so that the meniscus is at exactly 0 point. If insufficient blood is available for filling the entire hematocrit tube, the tube may be filled to any mark and the volumes, per cent of packed erythrocytes calculated by dividing the volume of packed erythrocytes by the total volume of blood in the tube. If the tube is filled to exactly the 0 mark, readings can be taken directly from the tube.

Close the open end of the tube with a small rubber stopper supplied with the apparatus to prevent evaporation. This is not necessary if the tube has been filled to the 0 mark. If less than this amount of blood is used, the open end of the tube must be closed to prevent evaporation during centrifugation.

At this point, if desired, the Wintrobe tube may be placed in a rack or in an upright position and allowed to stand for 1 hour to determine the sedimentation rate, and then centrifuged for the hematocrit determination. If the sedimentation rate is not desired, centrifuge for 30 minutes at 3,000 r.p.m.

Read the volume of packed red blood cells directly on the tube at the meniscus of the erythrocyte column. Read from the bottom up and multiply the reading on the right side (cm.) of the tube by 10 to obtain the volume per cent of packed erythrocytes.

Examine the hematocrit tube for other features. Beginning at the top of the tube, observe the **fatty layer**, which is normally barely visible. When the blood fat content is exceedingly high, a layer of several millimeters may be seen at the top of the tube. The main column in the tube from the top down is plasma. Normally, this **plasma layer** is a pale straw-colored yellow and is fairly clear. A cherry-red color is evidence of excessive hemolysis. Hemolysis may be due to faulty technic, such as collection of blood in wet syringes, wet needles, or placing it in a wet hematocrit tube. Hemolysis may also occur as a result of exposure of the blood to high temperatures (radiators) before the determination is made, or as a result of some inherent disease of the red blood cells. In the presence of excessive hemolysis, the hematocrit values will be abnormally low and, therefore, incorrect. In such cases, a new blood specimen should be obtained and all precautions taken to prevent hemolysis.

The plasma may also be tinted a deep yellow or almost brown color, indicating the presence of jaundice. The intensity of this color is an indication of the intensity of the jaundice present.

Just beneath the plasma layer is a reddish-gray layer or "**buffy coat**," which normally varies in thickness from 0.5 to 1.0 mm. This layer consists of packed leukocytes and platelets, the thickness of the layer roughly representing the total counts of leukocytes and platelets. If this layer is exceedingly thin, e.g., less than 0.5 mm., it may indicate a reduction in platelets or in leukocytes. If it is greater than 1 mm., it usually represents an increase in the leukocyte count above 10,000 per cu.mm. Sometimes this layer may subdivide into two indistinctly separated layers, the uppermost portion of which is rich in platelets and the lower almost exclusively leukocytes. In cases of marked leukopenia, the leukocyte layer may be aspirated and spreads made for differential counting in order to obtain a greater concentration of white cells. This is not recommended as a routine procedure because white cells collected in any anticoagulant show morphologic changes sufficiently great to give a wrong impression of the true character of these cells.

In searching for **lupus erythematosus** ("**L.E.**") cells, the buffy coat of the hematocrit tube serves as a very useful concentration method.

Permit the blood specimen to remain at room temperature for approximately 2 hours before centrifugation, if the preparation is to be examined for L.E. cells.

Some workers who collect **bone marrow** preparations in an anticoagulant use the hematocrit tube method as a rough estimate of the relative cellularity of the bone marrow. Various figures are given for the normal thickness of the buffy coat of bone marrow, ranging from 5 to 10 mm. This crude method is not a true gauge of the cellularity of the bone marrow, varying considerably with the technic of the operator who performs the aspiration and with the amount of bone marrow removed.

In the event that less than 1 c.c. of blood is available, the hematocrit determination made with this lesser quantity is calculated as follows:

Suppose that the hematocrit tube has been filled to the 6.0 mark (60 mm.). The volume of packed red cells after 30 minutes' centrifugation is at the 2.4 mark (24 mm.).

$$\text{Volume of packed red blood cells (per cent)} = \frac{24}{60} \times 100 = 40\%.$$

(II) Microtechnic for Hematocrit Determination

Equipment.—

Capillary tubes, plain or heparinized.

High-Speed Centrifuge.—The Guest-Weichselbaum* centrifuge is excellent. This centrifuge is made with a motor which has a speed of 10,000 r.p.m., and has an automatic electric timer built into the apparatus. The special flat head contains notches which permit handling 24 microhematocrit determinations simultaneously. A flush-fitting cover, held in place by a set screw, is furnished with the centrifuge. See Fig. 159.

A plastic hematocrit linear scale for direct reading is shown in Fig. 163.



Fig. 159.—The International microhematocrit centrifuge, showing special hematocrit head, cover, and automatic timing device. (Courtesy International Equipment Co., Boston, Mass.)

Technic.—

If oxalated venous blood is available, fill the open end of a *plain* capillary with blood. If capillary blood is used, fill a *heparinized* capillary tube with the blood sample.

Seal the ends of the tube with a lighted match or a burner.

Place the capillary in the centrifuge, the sealed end toward the outside, taking care to balance it against another tube of the same size on the opposite side of the centrifuge head.

Place the cover on the centrifuge and centrifuge, setting the automatic timer at 4 minutes.

*Made by the A. S. Aloe Company, St. Louis.

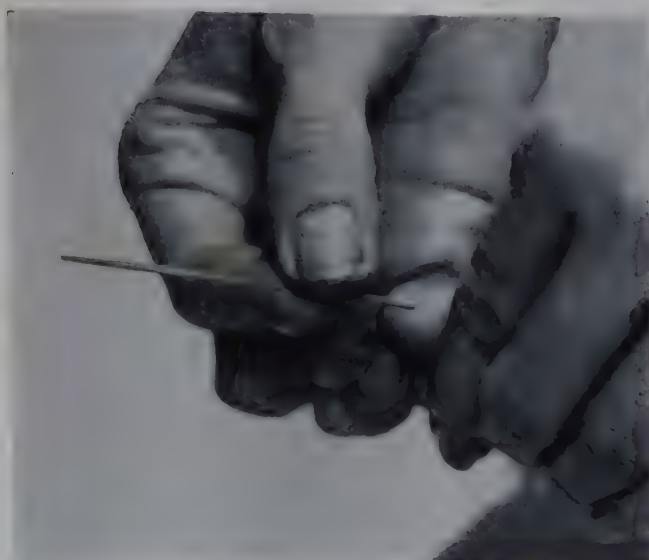


Fig. 160.—Obtaining finger blood for the microhematocrit test. The blood is taken in a capillary tube containing an anticoagulant. (Courtesy Aloe Scientific, St. Louis, Mo.)



Fig. 161.—Sealing the capillary containing blood preparatory to centrifuging in a special hematocrit-head centrifuge. (Courtesy Aloe Scientific, St. Louis, Mo.)



Fig. 162.—Placing the sealed capillary in the special centrifuge head for the microhematocrit test. Tubes must be placed opposite each other for balancing the centrifuge head. (Courtesy International Equipment Co., Boston, Mass.)

Read the per cent of blood cell volume directly from the graphic reader. Hold the tube against the linear chart so that the top of the liquid is exactly at the top line and the bottom of the tube against the bottom line. Move the tube until it reaches such position, then read the volume of packed cells directly from the reader.

With a magnifying hand lens, examine the characteristics of the plasma and those of the buffy coat of platelets and leukocytes.

Comment: The advantage of this micromethod is that it requires small quantities of blood and therefore lends itself well to pediatric practice. In emergency situations such as those associated with blood loss and shock, the short centrifuging time offers a distinct advantage over the macrotechnic. Another advantage is that the capillary tubes are inexpensive and disposable.

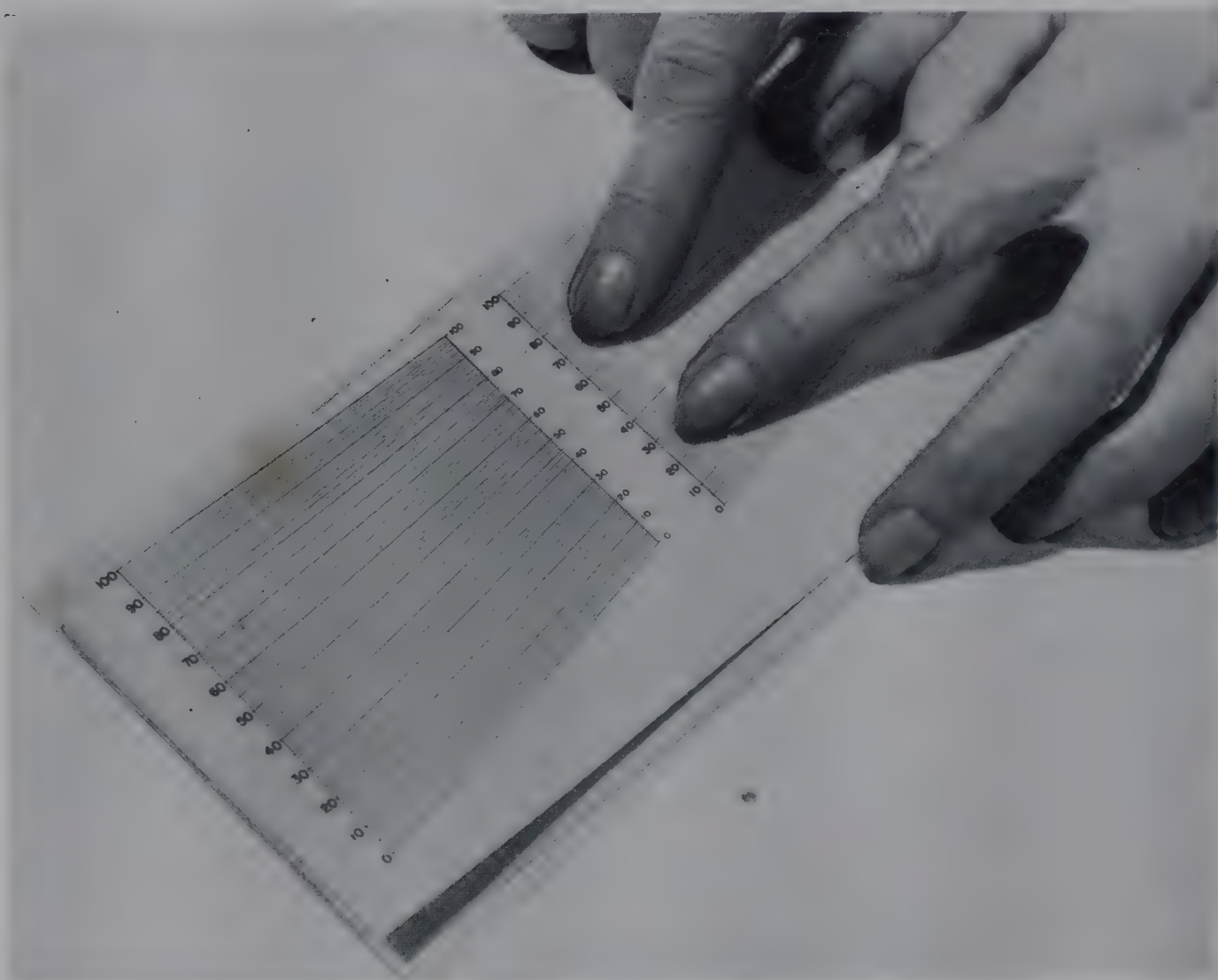


Fig. 163.—Special plastic hematocrit linear scale for reading cell volume per cent of erythrocytes in the microhematocrit test. The capillary tube is placed on the scale with the bottom of the blood column on the "0" line, the top of the column of blood at the "100" line. Read the volume of packed cells from the scale at the left of the graphic reader. (Courtesy Aloe Scientific, St. Louis, Mo.)

Interpretation.—

In anemias generally, the hematocrit is reduced below the normal figures, that is, below 40 volumes per cent in males and 37 in females. This method is therefore a very simple, readily available method for detecting the presence or absence of anemia. However, the hematocrit alone does not disclose the nature of the anemia and at times may fail to reveal an existing anemia, although this is rarely the case.

In polycythemia, the hematocrit value is elevated above 54 in males and 47 in females. The degree of increase, however, does not indicate the nature

of the polycythemia, failing to distinguish between polycythemia vera and secondary polycythemia.

In shock, associated with surgery, trauma, and burns, hemoconcentration occurs and may be measured by increases in the hematocrit values (rapidly rising).

In conditions associated with hydremia, such as cardiac decompensation, excessive administration of fluids, and other similar states, the hematocrit values may be below normal, and yet no anemia be present.

The hematocrit test is necessary for determination of some of the erythrocyte constants.

ERYTHROCYTE CONSTANTS OR INDICES

Having determined the erythrocyte count, hemoglobin, and hematocrit value in a patient, it is possible to express individual aspects of the red blood corpuscles in either the absolute terms of Wintrobe¹ or in the older traditional relative terms. The most commonly used constants are helpful in designating such characteristics of the red blood cells as size and hemoglobin content, and hemoglobin concentration. In order for these constants to have any real significance, the basic determinations of erythrocyte count, hemoglobin, and hematocrit must be performed accurately. Wintrobe recommends the use of absolute terms for these constants in contrast with the older traditional terms which were relative and were based on percentages.

1. Constants Relating to Erythrocyte Size

(A) MEAN CORPUSCULAR VOLUME (ABSOLUTE CONSTANT)

The mean corpuscular volume (M.C.V.) in cubic microns ($c\mu$) is determined by the following formula:

$$\text{M.C.V.} = \frac{\text{volume packed red cells, c.c. per 1,000 c.c. of blood}}{\text{Erythrocyte count in millions per cu. mm.}}$$

Normal Values:

Adult males: average 87 $c\mu$, range 70 to 94.

Adult females: average 87 $c\mu$, range 74 to 98.

Examples.—

Normal Blood: Hematocrit is 45 volumes per cent, erythrocyte count is 5,000,000.

$$\text{M.C.V.} = \frac{45 \times 10}{5} = 90 \text{ } c\mu.$$

Microcytic Anemia: Hematocrit is 22.5, erythrocyte count 3,500,000. M.C.V. =

$$\frac{22.5 \times 10}{3.5} = 64 \text{ } c\mu.$$

Macrocytic Anemia: Hematocrit is 21.2, erythrocyte count, 2,000,000. M.C.V. =

$$\frac{21.2 \times 10}{2.0} = 106 \text{ } c\mu.$$

(B) VOLUME INDEX (RELATIVE CONSTANT)

This constant corresponds to the mean corpuscular volume. It is determined by the following formula:

$$\text{Volume Index} = \frac{\% \text{ volume packed erythrocytes}}{\% \text{ of erythrocytes}}.$$

Normal Values: Average, 1.0, range 0.85 to 1.15.

¹Wintrobe, M. M.: Clinical Hematology, Philadelphia, 1952, Lea & Febiger.

It is customary to take an arbitrary figure, such as 43.2, for the normal hematocrit, and 5,000,000 for the normal red blood cell count to represent 100 per cent in these calculations. As with all such determinations, there is a difference of opinion as to what constitutes 100 per cent. We suggest, for consistency, that the mean average levels given below be taken for these determinations:

	HEMATOCRIT	ERYTHROCYTES PER CU. MM.
Adult males	47	5,400,000
Adult females	42	4,800,000

Examples.—

Normal Female: Hematocrit, 40. $40 \div 42$ is 95% of normal. Red blood count, 4,500,000. $45 \div 48$ is 94% of normal. Volume index = $\frac{95}{94} = 1.01$.

Normal Male: Hematocrit, 48. $48 \div 47 = 102\%$ of normal. Red blood count, 5,200,000. $52 \div 54 = 96\%$ of normal. Volume index = $\frac{102}{96} = 1.06$.

Microcytic Anemia, Male Subject: Hematocrit, 22.5. $22.5 \div 47 = 48\%$ of normal. Red blood count, 3,500,000. $35 \div 54 = 65\%$ of normal. Volume index = $\frac{48}{65} = 0.74$.

Macrocytic Anemia, Female Subject: Hematocrit, 21. $21 \div 42 = 50\%$ of normal. Red blood count, 2,000,000. $20 \div 48 = 42\%$ of normal. Volume index = $\frac{50}{42} = 1.18$.

(C) COMMENTS ON THE DETERMINATION OF THE SIZE OF RED BLOOD CELLS

We recommend determination of the mean corpuscular volume over determination of volume index as an expression of the *mean* size of red blood cells. Certain reservations should be made, however, in accepting mean corpuscular volume figures as necessarily reflecting extreme accuracy. The accuracy of the mean corpuscular volume is only as great as the accuracy of the methods by which the red count and the hematocrit value have been determined.

There are many ways of determining size of the red cells. Perhaps the simplest is to examine a well-prepared blood spread, where recognition of variations in size as well as the general trend in size is readily perceptible. The blood spread should be used as a check on mean corpuscular volume determinations.

There are other methods for determining size of the red blood cells, but, generally speaking, these are of relatively little clinical practical value. Most of these are perhaps of greater historic interest than of practical value. Among the earliest attempts at the measurement of the actual diameter of red blood cells is the method of Price-Jones,¹ who made camera lucida drawings of red cells, measuring the actual diameters of the drawings of from 500 to 1,000 cells. From these drawings, he constructed curves which are spoken of as the "Price-Jones curves," showing the distribution of various cell sizes. These curves were so graphed that shifts to the left of the normal distribution curve were seen when the mean diameter of the red cells was diminished, and shifts to the right were seen when the mean diameter was increased.

As a substitute for the camera lucida drawings there have been devised micrometer disks which can be inserted into the ocular system of a microscope and direct measurement of erythrocytes made with the scale on the micrometer disk. These measurements may be made on a dry stained blood film or on a wet preparation in the counting chamber, there being some shrinkage in the diameter of the red blood cells as seen in the fixed

¹Price-Jones, C.: Oxford Medical Publications, London, 1933.

preparation when compared with the wet. In dry preparations, the average red cell diameter is 7.5μ , with a range of 7.2μ to 7.8μ . In wet preparations, the erythrocyte diameter is 8.4μ , with a range of 7.4μ to 9.4μ . Cells smaller than the lower values in the range are considered microcytic, and those larger than the upper figures, macrocytic.

A number of methods have been devised based on the Pijper principle² which consists essentially of the deflection of white light when passing through a screen to form a spectrum. Halometers have been produced in Europe over a good number of years, and within relatively recent years Haden³ produced an instrument based on this principle, which is commercially available. Dingee⁴ suggested a method originally described by Pryce.⁵ In her ingenious technique, two 25-watt frosted electric light bulbs were placed 28 inches apart on a wall against a black background. A tape measure was fixed between the lights and was held to a stained blood film. Upon looking through the blood spreads toward the two lights, two halos will be seen. The distance between the blood film and the light sources is changed until the red innermost circles of the spectrum of the two circles touch. The distance in feet from the lights to the blood film is then determined and this distance corresponds to the mean diameter in μ of the red blood cells.

A particularly good habit for all technicians is to make an examination of the relative size and distribution of the red blood cell population in making routine blood counts.

2. Constants Related to Hemoglobin Content of Erythrocytes

(A) MEAN CORPUSCULAR HEMOGLOBIN (ABSOLUTE CONSTANT)

The mean corpuscular hemoglobin indicates the weight of hemoglobin per average erythrocyte. The Whitrobe formula for computing mean corpuscular hemoglobin is as follows:

$$\text{M.C.H. in micromicrograms (}\gamma\gamma) = \frac{\text{Hemoglobin in gm. per 1,000 c.c. blood}}{\text{Red count in millions per cu. mm.}}$$

Normal Values:

Adult Males: 29 micromicrograms average; range, 25 to 34.

Adult Females: 29 micromicrograms average; range, 24 to 33.

Example.—

Red count, 5,000,000; hemoglobin, 15.6 gm. per 100 c.c.

$$\text{M.C.H. (}\gamma\gamma) = \frac{15.6 \times 10}{5.0} = 31.2.$$

(B) COLOR INDEX (RELATIVE VALUE)

This determination corresponds to the mean corpuscular hemoglobin. Formula:

$$\text{Color Index} = \frac{\% \text{ hemoglobin}}{\% \text{ erythrocytes}}$$

Normal Values: Normal color index average, 1.0; range, 0.85 to 1.15.

In the formula, 5,000,000 erythrocytes per cu. mm. is usually considered 100 per cent. As with the determination of volume index and for the sake of consistency, we recommend that the following values be used as 100 per cent in calculating color index:

	HEMOGLOBIN	ERYTHROCYTES PER CU. MM.
Adult males	15.8 gm. %	5,400,000
Adult females	13.9 gm. %	4,800,000

Examples.—

Male Subject: Hemoglobin, 14.6 gm. %. $14.6 \div 15.8 = 92\%$ of normal. Erythrocytes, 4,800,000. $48 \div 54 = 89\%$ of normal.

$$\text{Color index} = \frac{92}{89} = 1.02.$$

²Pijper, A.: J. Lab. & Clin. Med. 32: 857, 1947.

³Haden, R. L.: J. Lab. & Clin. Med. 25: 399, 1940.

⁴Dingee, J. E.: Lab. Digest 5: 8, 1941.

⁵Pryce, D. M.: Lancet 2: 275, 1929.

Female Subject: Hemoglobin, 4.5 gm. %. $4.5 \div 13.9 = 32\%$ of normal. Erythrocytes, 2,100,000. $21 \div 48 = 45\%$ of normal.

$$\text{Color index} = \frac{32}{45} = 0.71.$$

(C) COMMENT ON MEAN CORPUSCULAR HEMOGLOBIN DETERMINATION

The mean corpuscular hemoglobin generally parallels changes in the mean corpuscular volume. In other words, when the red cells are smaller, the amount of hemoglobin per red cell is less. When the red cells are larger, the amount of hemoglobin is greater. But in both instances, the actual *concentration* of hemoglobin within the red cells may well be the same. In view of this, determination of the mean corpuscular hemoglobin is of relatively little value and adds very little to the knowledge already gained by determination of the mean corpuscular volume. Wintrobe stresses a determination which he feels is much more significant, the mean corpuscular hemoglobin concentration.

(D) MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (ABSOLUTE CONSTANT)

Mean corpuscular hemoglobin concentration expresses the concentration of hemoglobin per erythrocyte.

Normal Values: The normal values for mean corpuscular hemoglobin concentration (M.C.H.C.), expressed in per cent, is 34% for both males and females, with a range of $\pm 2\%$.

The formula for determining M.C.H.C. is:

$$\text{M.C.H.C. (\%)} = \frac{\text{Gm. hemoglobin per 100 c.c. blood}}{\text{Volume \% of erythrocytes (hematocrit)}} \times 100.$$

Examples.—

Normal Patient: Hemoglobin, 15.3 gm. %; hematocrit, 45.

$$\text{M.C.H.C.} = \frac{15.3}{45} \times 100 = 34\%.$$

Hypochromic Anemia: Hemoglobin, 8.6 gm. %, hematocrit, 36.

$$\text{M.C.H.C.} = \frac{8.6}{36} \times 100 = 24\%.$$

(E) COMMENTS ON MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION

Values below 30 per cent of the mean corpuscular hemoglobin concentration are indicative of the *hypochromic* anemias. Values above 36 volumes per cent are not seen because there is no known condition in which the *concentration of hemoglobin per unit volume* is greater than that which occurs in the normal red blood cell. Therefore, when the term “*hyperchromic*” is used, it cannot be in the sense that the concentration of hemoglobin in the red blood cell has increased. Wintrobe, as well as others, decries the use of the term hyperchromic. When used in this limited sense of a greater concentration of hemoglobin per unit volume, the term is definitely a misnomer. However, when used to describe red blood cells as seen in the stained blood film, the term “hyperchromic” is acceptable because these cells do appear to have a greater color than normal red blood cells. This more intense coloring is due to an increased thickness of the red blood cells rather than to an increase in concentration per unit volume of the cells. When the term is understood in this manner there is perhaps some justification for the continued use of this highly deceptive term.

The hemoglobin concentration of the red blood cell is readily apparent in properly stained blood films. Examination of such films should be made, to serve as a check on the determination of mean corpuscular hemoglobin concentration. If one examines enough hematologic material, there will be a fair number of instances in which the blood spread reveals red cells that appear mildly hypochromic, whereas the mean corpuscular hemoglobin concentration is normal, and vice versa. However, where there is a marked change in the concentration of hemoglobin in red blood cells, this will be apparent both in the blood film and in the mean corpuscular hemoglobin concentration determination. These two procedures should complement one another in the examination of a blood specimen.

(F) SATURATION INDEX (RELATIVE VALUE)

Saturation index corresponds to the mean corpuscular hemoglobin concentration.

Formula: $\text{Saturation index} = \frac{\% \text{ hemoglobin}}{\% \text{ hematocrit}}$

Normal Values: Average, 1.0; range, 0.85 to 1.15.

In this calculation the problem again arises as to which values should be used for the 100%. For the sake of consistency, we recommend the following:

	HEMOGLOBIN	HEMATOCRIT
Adult males	15.8 gm. %	47 vol. %
Adult females	13.9 gm. %	42 vol. %

Examples.—

Normal Male: Hemoglobin, 15.3 gm. %; $15.3 : 15.8 = 97\%$ of normal. Hematocrit, 45. $45 \div 47 = 96\%$ of the normal.

Saturation index $= \frac{97}{96} = 1.01$.

Hypochromic Anemia, Female Subject: Hemoglobin, 8.6 gm. %. $8.6 : 13.9 = 62\%$ of the normal. Hematocrit, 36. $36 \div 42 = 86\%$ of the normal.

Saturation index $= \frac{62}{86} = 0.72$.

Summary on Erythrocyte Constants.—

From a clinical standpoint, studies of mean corpuscular volume and mean corpuscular hemoglobin concentration in conjunction with well-prepared stained blood spreads can give inestimable information concerning the anemias. The other constants and indices are interesting but add very little to this. For speed in calculating the absolute constants, Wintrobe¹ presents a nomograph prepared by Robert E. Mason.

RETICULOCYTES

Reticulocytes are immature erythrocytes which show a reticulum *when stained by some supravital method*. They cannot be demonstrated in a blood spread stained in the routine manner. Reticulum may be seen in red blood cells both with or without nucleus.

The normal reticulocyte count is 0.5 to 1.5 per cent. An increase in reticulocytes is evidence of active regeneration of erythrocytes. Reticulocytosis

¹Wintrobe, M. M.: Clinical Hematology, Philadelphia, 1952, Lea & Febiger, p. 330.

of considerable magnitude follows adequate treatment of patients with pernicious anemia.

For such a relatively simple procedure, it is amazing how many variations there are for performing this test. We shall present several methods with the admonition that features of any of these procedures may be combined to form another method.

Method I. Method of Choice¹

Reagent.—

New Methylene Blue N Solution.—

Dissolve 0.5 gm. new methylene blue N

1.4 gm. potassium oxalate

0.8 gm. sodium chloride

in 100 c.c. distilled water.

Filter just before use.

Blood Specimen.—Venous oxalated blood can be used, if used within 2 to 3 hours after withdrawal from patients. Peripheral blood can also be used.

Technic.—

If venous blood is used, add 3 or 4 drops of methylene blue N solution to an equal quantity of well-mixed blood in a small test tube, and mix.

If peripheral blood is used, draw the methylene blue N solution into a white counting pipette until the bulb is half full, admit a small air bubble into the stem, and then draw up the blood from a fresh puncture until the bulb is filled. Mix.

Allow to stand 5 minutes.

Place 1 drop of the dye-blood mixture on the end of a clean glass slide, and spread in a manner similar to that for preparing a blood film. Make several slides.

Dry rapidly by waving slides in the air vigorously.

The slide may be examined under oil immersion without further preparation, or it may be counterstained with Wright stain, or Wright-Giemsa combination (see page 621).

Counting Reticulocytes.—

Using the four-field meander method of counting, count 1,000 red blood cells (250 per field), simultaneously enumerating those erythrocytes containing a reticulum (reticulocytes).

Calculation.—

$$\text{The reticulocyte count in per cent} = \frac{\text{No. reticulocytes counted}}{\text{No. of red blood cells counted}} \times 100.$$

Example.—

46 reticulocytes counted; 1,000 erythrocytes counted.

$$\text{Per cent reticulocytes} = \frac{46}{1,000} \times 100 = 4.6\%$$

Method II. Method of Cook, Meyer, and Tureen

Reagents.—

Solution A.—

1% Solution of Brilliant Cresyl Blue in Saline.

Dissolve 1 gm. of brilliant cresyl blue in 100 c.c. of warmed 0.85% sodium chloride.

When cool, filter into a glass-stoppered bottle.

Solution B.—

1% Solution of Potassium Oxalate in Saline.

Dissolve 1 gm. of potassium oxalate, neutral, in 100 c.c. of 0.85% sodium chloride.

Filter into a glass-stoppered bottle.

¹Lab. Digest 16: p. 6, 1952.

Technic.—

Place 5 drops solution A in a watch glass.

Add 1 drop solution B, and mix.

Make the puncture of the skin in the usual manner.

Using a Sahli pipette, or capillary tube, draw up some blood and an equal amount of the mixed stain. *To avoid precipitates, do not use an excess of the stain.*

Mix thoroughly by alternately blowing out onto a glass slide and drawing up into the pipette. This should be done several times to insure a good stain.

Allow to stand 5 or 6 minutes, covered with a Petri dish to prevent evaporation.

Place a small drop of the mixture of blood and stain upon the end of a glass slide and spread with a cover slip in the manner of making ordinary blood films.

Dry quickly to prevent distortion of the cells.

This slide may be examined immediately, or counterstained with either Wright or Giemsa method. If the Giemsa stain is used, the slides are fixed for 3 minutes in methyl alcohol preparatory to staining.

Proceed as in Method I, p. 604.

Comment: Although Cook, Meyer, and Tureen used the classical brilliant cresyl blue, the newer dye, new methylene blue N, could be substituted for it.

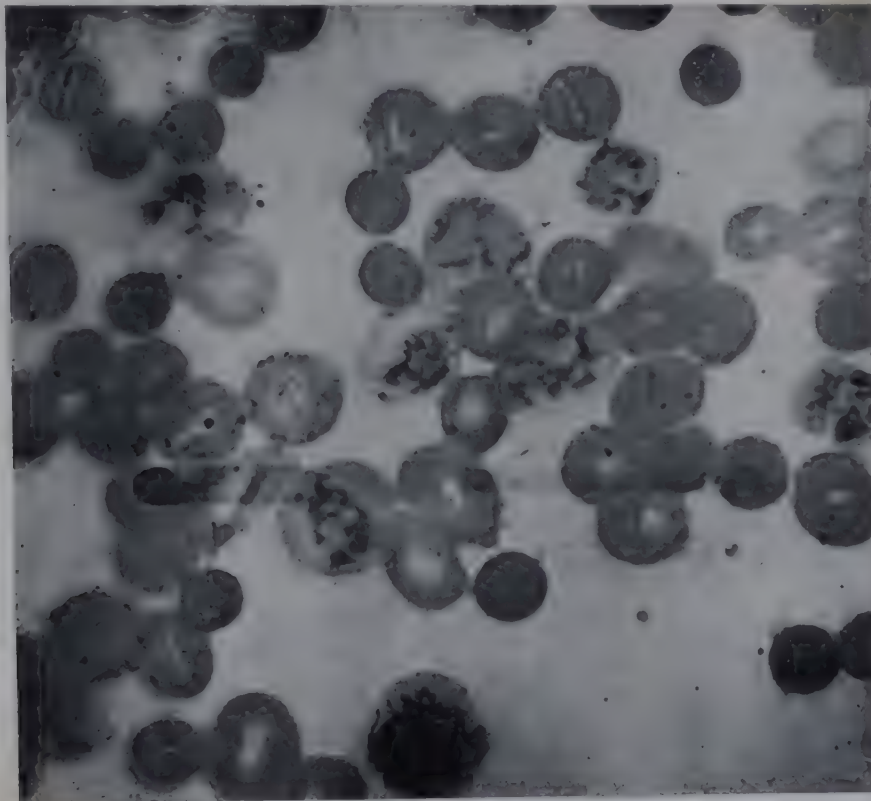


Fig. 164.—Hemolytic icterus, blood film, supravital stain, showing special staining of reticulocytes. ($\times 950$.)

Method III. Rapid Method of Schilling

This method is not permanent.

Reagent.—

1% Alcoholic Solution of Brilliant Cresyl Blue.—

Dissolve 1 gm. brilliant cresyl blue
in 100 c.c. absolute alcohol, grinding the dye in a mortar.

Filter after 24 hours.

Keep in a tightly stoppered bottle with a glass rod attached.

Technic.—

Dip the glass rod into the solution. Spread a thin layer over a fat-free slide, and allow to dry in the air.

Puncture the patient's skin in the usual manner, and place a very small drop of blood upon the layer of dye.

Place a large cover glass over the drop of blood. The drop of blood must be so small that it does not spread to the edges of the cover glass.

Examine with oil immersion objective after about 10 minutes.

Proceed as in Method I, page 604.

Method IV. Stitt Modification of Klemperer Method^{1, 2}

Materials Required.—

Stock Stain.—

1% Azure II in absolute alcohol.

Working Stain.—

Use 1 c.c. of stock stain to 5 c.c. of absolute alcohol.

Preparation of Slides.—

Thoroughly clean glass slides, which must be grease-free.

Place working stain in a suitable receptacle in which the grease-free slides may be dipped with a slow even motion, to obtain a thin, uniform surface of dye on the slide.

Allow the slides to dry in the air. Slides may be prepared in quantity and stored in dust-free slide boxes.

Technic.—

Place a very small drop of blood on a clean cover slip and invert this on a stained slide prepared as above. The weight of the cover slip should be sufficient to give an even spread of cells.

Examine under oil immersion.

Proceed to enumerate reticulocytes as in Method I, above.

Method V. Sabin³

Materials.—

Stock Solutions.—

Neutral red, special for supravital staining, prepared by dissolving 125 mg. neutral red in 50 c.c. of neutral absolute ethyl alcohol.

Janus green, special for supravital staining, is prepared by dissolving 125 mg. of the dye in 62.5 c.c. neutral absolute alcohol.

Working Solution.—

Dilute 1.1 c.c. of stock solution of neutral red in 10 c.c. of neutral absolute ethyl alcohol.

Add 8 drops of stock solution of Janus green.

Preparation of Slides.—

Flood grease-free clean slides with the working solution of Janus green-neutral red solution.

Drain off excess dye into working solution bottle.

Wipe off every drop of solution on dependent portion of slide.

Dry quickly by rapidly waving slide in the air or by holding it over a gentle flame.

Mark stained side of slide.

Slides prepared in this manner may be stored for future use, provided this storage is free from dust. Stained films must be thin, and must show even distribution of dye. Discard slides that are too thickly stained or that show uneven distribution of the stain.

¹Rear Admiral E. R. Stitt (ret.) U. S. N.: personal communication.

²Klemperer, G.: *Klinische Diagnostik*, 24 Auflage, p. 220, 1927.

³Sabin, F. R.: *Bull. Johns Hopkins Hosp.* 34: 277, 1923.

Sabin, F. R., Austrian, C. R., Cunningham, R. S., and Doan, C. A.: *J. Exper. Med.* 40: 845, 1924.

Sabin, F. R., Cunningham, R. S., Doan, C. A., and Kindwall, J. A.: *Bull. Johns Hopkins Hosp.* 37: 14, 1925.

Cunningham, R. S., Sabin, F. R., Sagiya, S., and Kindwall, J. A.: *Bull. Johns Hopkins Hosp.* 37: 231, 1925.

Technic.—

Place a small drop of blood on a clean cover slip and invert upon the stained surface of a neutral red-Janus green slide.

Seal edges of cover slip with petrolatum or a mixture of petrolatum and paraffin.

Allow preparation to stand 10 minutes before examining for reticulocytes. The slide may be examined up to 24 hours later if kept refrigerated at 4° C.

Examine under oil immersion.

Count reticulocytes as outlined under method I.

Examination of Sabin-Stain Slides for Supravital Staining of Leukocytes.—

Neutrophilic leukocytes (juveniles, "stabs," and segmented forms) move constantly with characteristic ameboid motion. The granules are numerous, small, pale red, and are constantly streaming through the cytoplasm. The nucleus usually is in the rear of the moving cell. There are present, also, one or more rounded bodies, presumably digestive vacuoles, which take the stain slowly. They indicate phagocytic activity, according to Sabin. They vary in color. Some leukocytes are round and motionless, the structureless nucleus nearly filling the cell, and the granules, although distinctly visible, not taking the stain. These are dead or dying neutrophils. During the middle of the day, the number of these dead neutrophils is greatest. They are increased by faulty technic.

Eosinophiles and basophiles show red granules. The granules of the basophiles are smaller than those of the eosinophiles, and are uneven in size and staining characteristics. There are no digestive vacuoles in either cell. Eosinophiles are actively motile; basophiles are sluggish.

Monocytes with oval nuclei are rounded and practically nonmotile. The very fine salmon-colored granules are grouped around a clear spot, the centrosphere. There are a variable number of larger red bodies, presumably digestive vacuoles. Those monocytes with lobulated or curved nuclei are irregular in shape, usually elongated, and are sluggishly motile. The red vacuoles usually displace or obscure the fine granules.

The **lymphocytes** show a clear cytoplasm with a few small vacuoles which take the red stain. When Janus green is added, a clump of blue mitochondria may be seen opposite the nucleus. The nucleus is oval or indented. There is no motility, although a few moderate-sized lymphocytes show sluggish motility.

Comment: Preparations of this kind are commonly referred to as "supravital." These may be used for differential counting and blood platelet estimations, as well as for reticulocyte counting. Some workers prefer this method to the fixed spread technic. One major objection is the lack of permanence of the preparations, so that preparations must be examined within 24 hours, and there are no slides available for future reference.*

Comments on Reticulocytes

An increase in the reticulocyte count is evidence of active regeneration of erythrocytes. The most classical reticulocytosis is that which occurs after the administration of erythrocyte maturation factor (liver extract or vitamin B₁₂) to patients with pernicious anemia in relapse. This reticulocytosis usually reaches a level of 20 per cent by the seventh to tenth day after initiating treatment, and then drops. In part, the height of response is governed by the degree of the anemia.

Patients with iron deficiency anemias, such as occur with blood loss, show reticulocytosis after iron therapy. The degree of reticulocyte response is usually less than that seen in pernicious anemia under treatment. Diseases associated with hemolytic crises, such as congenital hemolytic icterus, sickle cell anemia, and erythroblastosis fetalis, may show exceedingly high (40 to 60 per cent) reticulocyte counts.

*Permanent slides may be prepared from Method II.

Increase in Reticulocytes:*Physiologic*

At birth
Menstruation
Pregnancy

In Disease

All regenerative anemias
Blood intoxication
Erythroblastic anemias
Granulomatosis
Hemolytic anemia, very high
Following hemorrhage
von Jaksch's disease
Kala-azar
Lead poisoning, very high
Leukemia
Malaria
Pernicious anemia and sprue following liver therapy (as a response to treatment), and following vitamin B₁₂ therapy
Polycythemia vera
Relapsing fever
Sickle cell anemia
Splenic tumor
Frequently in trypanosomiasis
It has been observed in typhoid fever and brucellosis
Many parasitic infestations

Normal or Increased

Normal individuals
Progressive hypocythemia, not true aplastic anemia

Low or Absent

Idiopathic aplastic anemia
Acute benzol poisoning
Severe aregenerative anemias

Value of Reticulocyte Counts.—

1. To determine the response of the pernicious anemia patient to liver therapy. (See page 720.) Or to confirm a diagnosis of pernicious anemia by therapeutic response.
2. To aid in the diagnosis of lead poisoning and hemolytic icterus. (See pages 725 and 751.)
3. To determine whether or not regeneration of erythrocytes is proceeding normally, and if it occurs at all. This is of value in establishing a diagnosis of aplastic anemia, and in differentiating aplastic anemia from agranulocytosis and other conditions. (See page 748.)
4. To aid in the prognosis of acute hemorrhage, where an increase denotes regeneration of erythrocytes.

FRAGILITY OF ERYTHROCYTES**I. Osmotic (Saline) Fragility****A. Method of Fennel^{1, 2}****Materials Required.—****Stock Solutions of Sodium Chloride.—**

Prepare 0.30%, 0.38%, 0.45%, and 0.60% sodium chloride solutions in distilled water. Precision in preparing these stock solutions is absolutely essential. The sodium chloride must be chemically pure, dried, and weighed on an analytical balance. Solutions must be diluted to volume in accurate volumetric glassware.

¹Fennel, E. A.: *Am. J. Clin. Path., Tech. Sec.* 8: 21, 1944.

²Fennel, E. A.: *Lab. Digest* 8: 4, 1944.

Capillary or Venous Blood.—Venous blood may be heparinized or oxalated, or cells may be collected from clotted blood and suspended in their own serum.

Powdered Saponin.—

Photoelectric Colorimeter.—Use distilled water for “blank,” and green filter.

Chart prepared on semilogarithmic paper (Fig. 165).

Screening Procedures.—

Add 1 drop of blood to approximately 5 c.c. of 0.38% sodium chloride solution.

Mix well, stopper, and centrifuge for 5 minutes at 1,500 r.p.m. within the next few hours.

Carefully pipette off the supernatant fluid and transfer to a photoelectric colorimeter cuvette. Obtain the reading and record as “unknown.”

Return the supernatant fluid to the original tube.

Add a pinch of saponin, as much as goes on a flat toothpick, to the tube, stopper, and invert several times, and transfer to a colorimeter cuvette. Obtain reading and record as 100% hemolysis.

Determination of Percentage Hemolysis: Draw string A, Fig. 165, taut so that it crosses the colorimeter reading for the saponin-treated specimen (100% hemolysis). Then find where the colorimeter reading of the unknown crosses this line. Follow this reading across horizontally to obtain the per cent of hemolysis in the unknown.

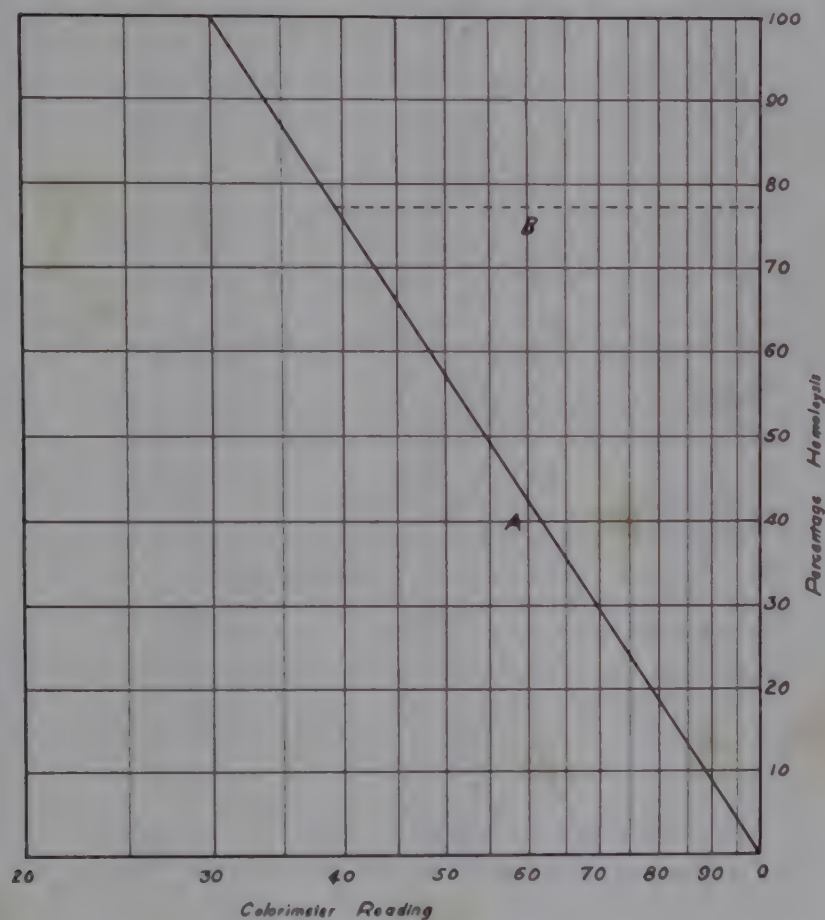


Fig. 165.—Chart to determine osmotic fragility of erythrocytes, method of Fennel. Saponin-treated specimen reads at 30 on the colorimeter (line A). Unknown reads at 40. Percentage hemolysis (B) is 76.

Example.—Saponin-treated specimen reads 30 on the colorimeter. Draw string to 30 on colorimeter scale on chart. Unknown reading is 40. This crosses string at level of B in Fig. 165. Extend this line to the right to obtain the per cent of hemolysis, which in this case is 76.

Interpretation.—At 0.38% concentration of saline there will be 70% to 95% hemolysis. If 95% or more hemolysis is present, this indicates increased osmotic fragility and a more elaborate test (see below) should be carried out. If there is less than 70% hemolysis, this indicates increased resistance and the complete procedure below must be carried out.

Comment: If the colorimeter cuvette requires more fluid, simply double or triple the amount of saline and blood. An extra quantity of saponin might be needed also.

Complete Procedure.—

Add 2 drops (about 0.06 c.c.) of blood to 6 c.c. of each strength of sodium chloride, 0.30%, 0.38%, 0.45%, and 0.50%.

Mix and decant one-half of each tube to another tube containing a pinch of saponin. Label the mates in these tubes.

Allow all tubes to remain for at least 30 minutes and centrifuge all tubes.

Transfer supernatant fluid of all tubes to photoelectric colorimeter cuvettes, and obtain readings for saponin-treated and unknown samples for each strength of saline.

Determine per cent of hemolysis for each strength of saline as indicated in the screening procedure above.

NOTE: Use mated samples only, e.g., the saponin-treated 0.38% saline sample against its matching half portion, etc.

Interpretation.—

Normal Ranges:

Concentration of sodium chloride	0.30%	0.38%	0.45%	0.60%
Per cent of hemolysis	95-100	85-95	30-50	2-6

Comment: Fennel has described adaptation of this method to a Duboscq type of colorimeter,^{1, 2} in which instance a different type graph is necessary.

B. Method of Sanford

Materials Required.—

Stock Solution of 0.5% Sodium Chloride.—

Accurately weigh 0.5 gm. sodium chloride on an analytical balance and dissolve in distilled water in a 100 c.c. volumetric flask. Dilute to 100 c.c. with distilled water and mix thoroughly.

Fresh Venous Blood, or 3:2 Oxalate Blood specimen from patient and from a normal control.

Series of 12 small test tubes in a rack, numbered 25, 24, 23, etc., down to 14. Arrange a duplicate series of tubes for control.

Capillary Pipette or Syringe with 19 to 21 gauge needle.

Preparation of Different Concentrations of Saline.—

Add to each tube the number of drops of 0.5% saline indicated by the number on the tube; e.g., to tube 25, add 25 drops of 0.5% saline, to tube 24, add 24 drops, etc. In making this transfer, use the same pipette or syringe, and hold at the same angle to insure uniformity of size of drops.

Rinse the pipette thoroughly with distilled water.

Add a sufficient number of drops of distilled water to each tube to bring the total volume in the tubes to 25 drops.

The percentage strength of saline in each tube may be obtained by multiplying the number on the tube by 0.02. For example, tube 16 has a concentration of 0.32%, etc. Mix all tubes thoroughly.

Technic.—

Add 1 drop of patient's blood to each tube in the series.

Prepare a duplicate set using normal blood.

Invert each tube to insure thorough mixing.

Allow tubes to remain at room temperature at least 2 hours. Some observers recommend incubation at 37° C., whereas others recommend refrigeration at 4° C. for longer time.

¹Fennel, E. A.: Am. J. Clin. Path., Tech. Sec. 8: 21, 1944.

²Fennel, E. A.: Lab. Digest 8: 4, 1944.

Examine the supernatant fluid for beginning hemolysis (faintest pink color) and for complete hemolysis (no sediment of erythrocytes remaining in the bottom of the tube). Gentle centrifugation (1,000 r.p.m. for 5 minutes) aids observation of supernatant fluid.

Normal Values: Hemolysis begins at 0.42% or 0.44% (tubes 21 and 22) and is complete at 0.34% saline (tube 17).

Comment.—With this technic, osmotic fragility above 0.5% saline is not included. If it is desirable to determine the fragility of erythrocytes in higher concentrations of saline, it is necessary to prepare saline solutions of higher concentrations. The Wintrobe method below considers this feature.

C. Wintrobe Modification for Osmotic Fragility¹

Materials Required.—

Stock Solution of 0.75% Saline.—Prepare with precision 0.75 gm. NaCl per 100 c.c.

Fresh venous or 3:2 oxalate blood specimen from patient and from normal control.

Series of 18 test tubes in a rack, numbered from 48 down to 14 in steps of 2; e.g., 48, 46, 44, etc., to 14.

Arrange a duplicate series of tubes for a control.

Serologic pipette, 5 c.c., graduated in 0.1 c.c.

Distilled water.

Preparation of Different Concentrations of Saline.—

Into each tube, add 0.75% saline solution in amounts equal to $\frac{1}{10}$ of the number on the tube; e.g., in tube 48, place 4.8 c.c. of saline, in tube 46, 4.6 c.c., etc.

To each tube add sufficient distilled water to bring the total to 5 c.c.

The concentration of sodium chloride in each tube will be $\frac{1}{50}X \times 0.75\%$, where X represents the number of c.c. of saline in the tube. In simpler terms, multiply the number on the tube by 0.015 to obtain the concentration of sodium chloride in the tube.

Example.—Tube 48 = $48 \times 0.015 = 0.72\%$ saline.

Mix all tubes thoroughly.

Technic.—

To each tube add 0.2 c.c. of patient's blood.

Prepare a similar series using normal blood as a control.

Shake tubes gently to insure thorough mixing.

Place in a refrigerator for 2 hours.

Examine the supernatant fluid of tubes for hemolysis as in the Sanford method (above).

Normal Values: Hemolysis begins at 0.45% to 0.39% saline; well marked in 0.42% to 0.36%; complete in 0.33% to 0.30%.

II. Mechanical Fragility²

Materials Required.—

Mechanical shaking device.³

50 c.c. Erlenmeyer flasks.

Glass beads 4.0 mm. in diameter.

Incubator.

Sterile applicators.

Centrifuge and hematocrit tubes.

Distilled water.

1.25% Sodium Chloride Solution.—

¹Wintrobe, M. M.: *Clinical Hematology*, Philadelphia, 1952, Lea & Febiger, pp. 161-162.

²(a) Shen, S. C., Castle, W. B., and Fleming, E. M.: *Science* **100**: 387, 1944.

(b) Young, L. E., Izzo, M. J., and Platzer, R. F.: *Blood* **6**: 1073, 1951.

(c) Gardner, F. H., McElfresh, A. E., Harris, J. W., and Diamond, L. K.: *J. Lab. & Clin. Med.* **37**: 444, 1951.

³Goldblood, R. B., Fischer, E., Reinhold, J., and Hsia, D. Yi-Yung: *Blood* **8**: 165, 1953.

Accurately weigh 1.25 gm. sodium chloride on an analytical balance, and dissolve in distilled water in a 100 c.c. volumetric flask. Dilute to 100 c.c. with distilled water.

Technic.—

Draw 5 c.c. of blood with sterile precautions.

Transfer to a sterile 50 c.c. Erlenmeyer flask containing glass beads and defibrinate by gentle rotation.

Divide the defibrinated blood into two parts. Use one part for immediate determination of mechanical fragility and incubate the other portion at 37° C. for 24 hours, then determine the mechanical fragility of this specimen.

Adjust the hematocrit of the defibrinated blood specimen to 35 volumes per cent by adding or removing plasma.

Preparation of Rotated Sample (S).—

Place 0.5 c.c. of adjusted sample in a 50 c.c. Erlenmeyer flask in the apparatus. The flask should contain 8 glass beads of 4.0 mm. diameter.

Rotate in a mechanical rotor at 100 r.p.m. for 60 minutes.

Take duplicate 0.1 c.c. samples of this rotated specimen and add each one to 1 c.c. of 1.25% sodium chloride solution in a centrifuge tube.

Preparation of Sample (H) Showing Complete Hemolysis.—

Add 0.1 c.c. of the adjusted blood specimen to 1 c.c. of distilled water in a centrifuge tube.

Preparation of Sample (O) Showing No Hemolysis.—

Add 0.1 c.c. of the adjusted blood sample to 1 c.c. of 1.25% sodium chloride solution in a centrifuge tube.

Centrifuge samples S, H, and O at 1,000 r.p.m. for 5 minutes.

Remove 0.5 c.c. of the supernatant fluid of each centrifuged specimen and add this to 9.5 c.c. of distilled water.

Determine the hemoglobin of each diluted specimen in a photoelectric colorimeter.

Calculation.—

$$\text{Percentage of mechanical fragility} = \frac{S - O}{H - O} \times 100$$

S = average of duplicated rotated specimens.

Results.—

	UNINCUBATED		INCUBATED	
	MEAN %	RANGE %	MEAN %	RANGE %
Newborns	7.1	1.1 to 11.5	13.4	3.7 to 21.6
Children	4.0	0 to 6.0	10.5	3.5 to 23.0
Adults	4.0	1.3 to 6.7	10.8	4.6 to 19.0

ROUTINE STAINING OF BLOOD FILMS

Preparation of Glassware for Making Blood Films

Slides and Cover Slips.—

The cover glasses recommended are $\frac{7}{8}$ inch wide, size "B," square.

All new slides and cover glasses must be cleaned thoroughly before use to render them fat-free. Make a weak solution of a detergent (Alconox, etc.) by using about half a teaspoonful of detergent to a pint of warm water. Admit the slides and cover glasses into this solution, one at a time, so that the entire surface of each is in contact with the wetting agent. Allow them to remain in the solution for a period of 15 minutes to a few hours. Do not leave them in too long because they become coated and cannot then be used.

Wash very thoroughly, preferably in running water, until all traces of the detergent have been removed. Place them in warm water and dry immediately using a soft cloth. Linen which has been washed many times makes an excellent towel for drying slides. Use enough pressure in drying the slides to remove any traces of fat which may remain on the slides. Have clean slides and cover glasses on hand at all times in the laboratory.

If slides have been used for oil immersion magnification, and are to be kept, dip them in xylol to remove the oil, then holding them at an angle blow down the slide, blowing the xylol to the bottom of the slides and dry the drop of xylol that remains. These slides can then be stored or filed for future reference.

If used slides are to be washed and re-used, remove the oil with a cloth containing a few drops of xylol, and clean in the detergent as directed above. It is recommended that slides should be used only once.

With this method no boiling, washing with soap, or using of ether and alcohol is necessary. Slides are clean and fat-free without further processing. Do not, however, allow the detergent to dry on the slides.

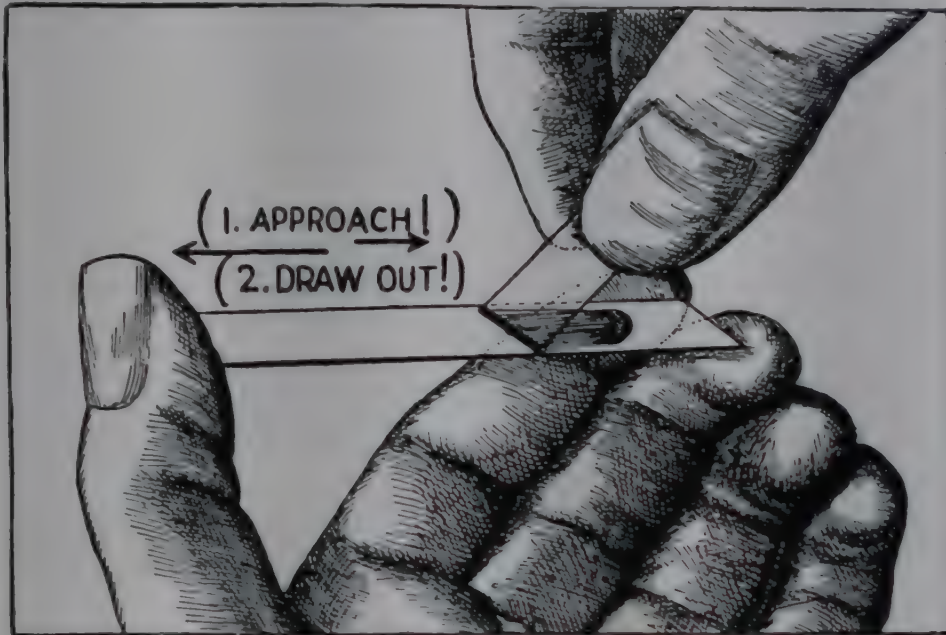


Fig. 166.—Correct method of making the film. (From Schilling: *The Blood Picture*.)

Blood Stains

Giemsa Stain.—Considerable difficulty has been encountered by many workers in the manufacture of a serviceable Giemsa stain. These difficulties have been overcome in our own laboratories so that we are now able to produce a stain certified by the Biological Stain Commission which gives uniform results, provided strict technic is followed in using this stain. The formula of R. D. Lillie has points of superiority over the original Giemsa stain, and we are now recommending its use.

Giemsa Stain, Formula of R. D. Lillie¹

Azure B Eosinate.—

Dissolve 10 gm. methylene blue, 85-88% dye content
in 600 c.c. distilled water in a mortar.

Add 6.8 c.c. of concentrated sulphuric acid sp. gr. 1.835 to 1.84.

Bring to a boil.

Add 2.5 gm. of potassium bichromate dissolved in 25 c.c. distilled water.

Boil 20 minutes.

Cool to 10° C. or lower. Place in the refrigerator overnight.

Add 17.5 gm. sodium bicarbonate slowly with frequent shaking.

Add a 5% solution of eosin Y of about 90% dye content, and shake constantly until the margin of the fluid appears pale blue or bluish pink. About 205 c.c. will be required. Three-fourths of this amount can be added at once.

Filter at once, preferably on vacuum funnel with hard paper.

¹Lillie, R. D.: *J. Lab. & Clin. Med.* 28: 1872-1875, Dec., 1943.

When fluid has been drawn through and the surface begins to crack, add 100 c.c. of distilled water, let drain, and wash again with a second 100 c.c. of distilled water.

Open out the filter paper and let it lie on a larger piece of filter paper or a paper towel and dry overnight on a warm plate or in the incubator at 37° C. The drying may be accelerated by using two 100 c.c. portions of acetone, or, preferably, 95% alcohol, as washes after the second wash with water. Drying at 55° to 60° C. has been tried, and produces quite a little alteration of the thiazin dye; less if acetone or alcohol washes are used and the heating limited to 2 or 3 hours. The resultant dye is the *crude azure B eosinate*.

Azure A Eosinate.—

Proceed as above for the azure B eosinate, but take 5 gm. of potassium bichromate in place of 2.5 gm., and dissolve it in 50 c.c. of distilled water.

Methylene Blue Eosinate.—

Dissolve 10 gm. methylene blue
in 600 c.c. cold distilled water.

Precipitate as before with 5% eosin, filtering and drying as above.

Methylene Blue.—

Use certified methylene blue chloride.

Solvent.—Equal parts of glycerin and methyl alcohol (certified) (below).

Lillie Stain.—

Grind the azure B eosinate, azure A eosinate, methylene blue eosinate, and methylene blue (when necessary) separately into fine powder, using separate clean mortars.

Weigh 840 mg. azure B eosinate

140 mg. azure A eosinate

420 mg. methylene blue eosinate

and 280 mg. finely ground methylene blue.

Mix the four powders thoroughly and pour over the surface of 200 c.c. of the solvent. in a brown bottle or flask. Cork the container.

Allow the powder to settle into the solvent gradually.

Shake frequently for 2 or 3 days, keeping the bottle between 50° and 60° C. between shakings.

If the bottle is tightly stoppered and the fluid level marked on the outside with a wax pencil or a piece of adhesive, there will be little or no loss from evaporation, and if there is, the fluid level can be restored by addition of methyl alcohol.

The glycerin should be neutral, anhydrous, and of the purest grade obtainable. Use methyl alcohol special for blood work.

Filter the stain through Whatman No. 5 filter paper into clean, dry, dark bottles. The stain is ready for use and is stable. Use as for Giemsa stain, original formula.

Wright Stain.—

(a) Wright Dye.—

0.5% Aqueous solution of sodium bicarbonate ----- 100 c.c.

Methylene blue ----- 1 gm.

Grind in a mortar until all the methylene blue is in solution.

Place in Erlenmeyer flask to a depth of not more than 6 cm.

Heat in an Arnold steam sterilizer at 100° C. for one hour.

Cool. Filter through filter paper. Save the filtrate.

To every 100 c.c. filtrate

Add 500 c.c. of a 0.1% aqueous solution of yellowish, water-soluble eosin (eosin Y).

Add the eosin solution slowly, stirring constantly, so as to form a precipitate. Test for a free eosin ring by placing a drop of the mixture on a piece of white filter paper and drying. The ring appears as a faint pink halo around a lavender spot. If the ring does not appear, add more 0.1% aqueous eosin until the ring forms. The center spot should be purple, not blue.

Filter through Whatman No. 5 filter paper, discarding the filtrate. Fold into 4 parts. Save the precipitate on the filter paper.

Dry the precipitate in an incubator. This may require several days.

This is the stock stain.

Place in a dark bottle, labelled "Wright Dye." This dye is stable.

(b) Wright Stain Solution.—

All glassware must be absolutely dry.

Dissolve 0.1 gm. Wright dye, above, in 60 c.c. methyl alcohol, acetone-free.

The dye must be ground in a mortar, and the alcohol added slowly until all the dye is in solution.

Place in a dark bottle.

Filter through filter paper after 24 hours.

The solution is not always stable.

Test, and label the bottle with the timing as determined by the test.

Preparation and Staining of Blood Films for Routine Differential Counts

Prepare the patient (page 572), producing hyperemia. Make the puncture, and discard the first one or two drops of blood.

Hold the slide between the thumb and middle finger of the left hand, and pick up a very small drop of blood with the slide so that it is about $\frac{1}{4}$ inch from the end. To avoid endothelial cells do not allow the slide to come in contact with the skin.

Hold the cover glass between the thumb and the first finger of the right hand. Approach the drop with the cover glass at an angle of 30 degrees. When the cover glass touches the drop of blood, allow the blood to run across the edge of the cover glass, then pull it across the slide to form a thin layer. Do not put any tension on the cover glass!

The film should not touch the edges of the slides; that is, it should be "margin-free." Scratch the patient's name in the center or the extreme end of the film as a permanent record. Let the films dry. In summer, cover the spreads to protect against flies, etc. Several spreads should be made at the same time.

We recommend the "margin-free" film because we have found that when the blood film is allowed to spread to the edge of the glass slide, the large cells tend to accumulate in groups at the edges, and the small cells, such as small lymphocytes and normoblasts, go to the center. With a combination of margin-free films and the four-field meander method of counting the cells, all spreads taken from the same individual at the same time by different individuals who know how to identify the cells should show the same differential count. The personal element, in other words, has been reduced to a minimum.

If the blood does not flow freely, remove the dried blood from the skin, gently wash the ear with a small amount of ether and allow to dry. If the blood does not flow freely now, a new puncture must be made. Never squeeze the ear to produce a flow of blood.

Do not use too large a drop of blood, or the spread will be too thick. Do not pull the drop too rapidly, as the film will tend to become thick. If the film is made too rapidly, it will not extend to the end of the glass slide.

Staining Blood Films

Giemsa Stain.*—

Steps in Technic Using Giemsa Stain:

Fix blood film in methyl alcohol 3 to 5 minutes.

Allow to dry.

Neutralize the distilled water.

Dilute the Giemsa stain, 1 drop for each c.c. of neutral water.

Cover slides with diluted stain for 30 minutes.

Tip up slides and wash with neutral distilled water.

Allow to dry in the air.

*Certified Giemsa stain is made by the Gradwohl Laboratories, St. Louis.

The stock Giemsa stain keeps indefinitely. The diluted Giemsa stain must be prepared just before staining. The container in which the stain is diluted must be free of acids and alkalies.

It is well always to dilute the stain in the same container, and, after using, to rinse the container thoroughly in tap water, then in neutral distilled water. The small amount of dye which adheres to the glass is not objectionable; in fact, the stain is better if made in a cylinder or flask which has been used for some time for Giemsa stain. The container should be cleaned from time to time to remove precipitates, after which it is neutralized.

Neutralizing Water in Staining of Blood Smears.—Neutral distilled water is to be used in the routine staining process. There has been inadequate stress on this subject in the various textbooks on clinical laboratory procedures. The author (R. B. H. G.) emphasized this in a monograph entitled, "Reaction of Water on the Staining of Blood Smears."¹ For instance, Todd and Sanford² state in connection with the staining with Wright's stain: "After one minute add to the staining fluid on the film the same quantity of distilled water by means of a second medicine dropper. This may be done by counting drops. Instead of distilled water it is much better to use the buffer solution of pH 6.4 described below. Blow gently on the diluted stain to make an even distribution, . . ." Furthermore, on the same page, they refer to McJunkin's³ article by suggesting "a buffer solution instead of distilled water in the second step of the procedure. This buffer solution with a pH of 6.4 is made by dissolving 6.63 gm. of monobasic potassium phosphate, and 3.20 gm. of dibasic sodium phosphate in one liter of distilled water. The phosphates should be recrystallized and the sodium phosphate should be exposed to the air for two weeks to lose its water of crystallization."

Kolmer and Boerner,⁴ in discussing methods for staining blood films, state in connection with the Wright stain that the following method, employing a buffer solution, is also recommended by Giordano: "(a) Buffer solution: 6.63 grams of acid potassium phosphate and 3.2 grams of dibasic sodium phosphate are dissolved in 1000 c.c. of distilled water." Nothing further is stated about the difficulties which will be encountered unless the reaction of the water is carefully controlled.

In Nicholson's⁵ book it is stated that the causes of poor results are precipitation on slide, evaporation, and scum. Also, another cause of poor results is lack of staining of the red cells and the nuclei of white cells. He also states: "If the red cells appear greenish instead of pink, the stain may be of too recent preparation or the glassware or distilled water may be alkaline. . . ." Further along he states: "If the red cells are buff or light pink and the nuclei of the white cells faint or unstained the fault is due to acid in the stain, on the slide or in the distilled water." He recommends rendering the water slightly alkaline under these circumstances, and that if this does not succeed the fault is in the stain and it will have to be discarded.

Osgood and Haskins⁶ in their book recommend the buffer phosphate suggested by McJunkin. They state that "in an emergency distilled water may be used instead of the buffer phosphate, but it is not nearly so satisfactory." On the same page they state: "If normal red cells stain bluish or greenish the fault is usually due to the use of water instead of phosphate, to contamination of the phosphate with alkali, or it may be due to overstaining and the time should be decreased."

We find in the fourth edition of *Laboratory Methods of the United States Army*,⁷ the following:

" . . . The exact time required for staining, and the pH of the water, must be determined for each lot of stain prepared but with a pH of 6.4 to 6.8 the interval given will be approximately correct.

¹Gradwohl, R. B. H.: J. Missouri M. A., Jan., 1936, pp. 14-16.

²Todd, J. C., and Sanford, A. H.: Clinical Diagnosis by Laboratory Methods, Philadelphia, 1935, W. B. Saunders Co., p. 261.

³McJunkin, F. A.: J. A. M. A. 74: 17-19, Jan. 3, 1920.

⁴Kolmer, J. A., and Boerner, F.: Approved Laboratory Technic, New York and London, 1931, D. Appleton & Co., p. 77.

⁵Nicholson, D.: Laboratory Medicine, Philadelphia, 1934, Lea & Febiger.

⁶Osgood, E. E., and Haskins, H. D.: Laboratory Diagnosis, Philadelphia, 1931, P. Blakiston's Son & Co., p. 634.

⁷Simmons, J. S.: Laboratory Methods of the United States Army, Philadelphia, 1931, P. Blakiston's Son & Co., p. 364.

"A pH of 6.8 is usually satisfactory. Thoroughly mix the two salts, as shown in Table 50, in a mortar and use 1 gm. to 2000 c.c. of distilled water. It may be necessary to vary the amounts of phosphates to obtain the desired results. Red is increased by lowering, and blue is decreased by raising the pH.

TABLE 50.—BUFFERED WATER (FOR USE WITH WRIGHT STAIN)

pH	Na ₂ HPO ₄ + 2H ₂ O	KH ₂ PO ₄
	gm.	gm.
6.2	1.816	9.504
6.5	2.723	8.316
6.8	4.539	5.940
7.0	5.447	4.752
7.2	6.355	3.564
7.4	7.262	2.376

"Microscopically, a properly stained blood smear will show red cells with a copper or tan color; lymphocytes with a pale blue cytoplasm and a dark blue nucleus; and neutrophils with a finely granular pink cytoplasm and a dark purplish-blue nucleus. If these characteristics are not distinct, prepare additional slides."

A simpler and better way of neutralizing water employs hematoxylin as an indicator. Water neutral to hematoxylin in our hands has proved better suited to staining blood cells than has water with a pH of 6.4. We have found that water which is neutral with the use of hematoxylin as an indicator has a pH of 6.8 to 7.0. This, therefore, gives a better approximate neutrality point than the buffer methods alluded to. The settlement of this neutrality point is especially necessary in dealing with spreads for the diagnosis of malaria, particularly thick spreads and thick drops. For instance, Barber and Komp¹ state in regard to the staining of plasmodia with the use of the thick drop: "Preliminary dehemoglobinization is unnecessary. Immediately before use dilute the Giemsa stock solution with distilled water of a pH 7.0 to 7.2."

Method for Neutralization of Water

Neutralize enough water for the day's work. The bottle used for storing the neutral water should preferably be Pyrex, or should be a bottle having been used for no other purpose than to hold distilled water. No rubber connections should be used on the still. If distilled water is purchased in large quantities, it should be poured, not siphoned, into the bottle that is to hold the neutralized water.

Pick up a few hematoxylin crystals with a pair of clean, dry forceps and place in a test tube. Rinse the tube with the water before testing because the tube might not be neutral. Pour approximately 5 c.c. of the water to be neutralized into the test tube containing the hematoxylin crystals, shake, and note the color. If the water is neutral, it will become a pale pink within 10 seconds. If the water is acid, it becomes yellow and remains yellow for longer than 5 minutes. If it is alkaline, it becomes reddish-purple immediately, or before 1 minute. If the water is acid, as it usually is, 1% potassium carbonate is used as a neutralizing agent. If it is alkaline as it comes from the still, it should not be used. Add the neutralizing agent, a drop at a time, to the stock bottle of water to be neutralized, shaking after the addition of each drop or two, and again testing the reaction. When the water turns pink without a trace of yellow or blue in 10 seconds it is neutral and may be used. If too much potassium carbonate has been added, the water becomes alkaline. Pour out some of this alkaline water, and add the slightly acid non-neutralized water. Proceed as before until the water is neutral.

Water handled in this manner gives excellent staining results with Giemsa or Wright stain. Staining characteristics of blood cells are altered with changes in the reaction of the water.

If the water is acid, erythrocytes are bright red orange instead of light yellowish orange. All eosin stains become very bright reddish orange. The eosinophiles stand out particularly brilliantly. In a count of eosinophiles alone it is advisable to use the

¹Public Health Report, 44, 1929.

naturally acid distilled water. The nuclei of all cells will stain a pale sky blue. The cytoplasm of all lymphocytes and monocytes is very pale blue. These two cell types cannot be differentiated from each other if the water is acid. The neutrophilic cells become extremely pale, the nuclei staining either pale blue or pale lavender, and the cytoplasm a definite pink. The age of the various neutrophiles cannot be determined accurately because of indefinite staining of the nuclei thus rendering a Schilling count practically an impossibility. Blood platelets are very pale blue. Malarial parasites lose much of their color, the red chromatin staining very pale and the blue cytoplasm being scarcely visible.

With alkaline water, erythrocytes stain blue or green. A purple cast is imparted to all blues in the preparation. The cytoplasm of lymphocytes becomes gray or lavender and cloudy, the cytoplasm of monocytes is decidedly pinkish lavender or even purple, the cytoplasm of the neutrophiles, deep lavender or almost red. Eosinophiles show deep gray or blue granules. The granules of all neutrophiles become very intensely overstained and appear more numerous and larger than normal. This makes the detection of toxic granules impossible. Chromatin granules of all nuclei assume the appearance of large black clumps. The identification of any cells having nucleoli becomes extremely difficult. Erythrocytes not only change in staining characteristics but also seem to have no center and frequently have horny edges so that all the cells look crenated. Most leukocytes look frayed at the edges. Malarial parasites stain particularly well, but if the water is too alkaline, it is impossible to differentiate the chromatin from the cytoplasm. Blood platelets swell and stain reddish lavender. One cannot identify them by their structure but only by their size and arrangement.

Another danger in staining blood films is in **using too little of the staining solution**, or in **overwashing**. Water will decolorize the stain. Slides must be washed very slightly, then allowed to stand on end to dry. In staining, the slides should be placed on a staining bridge over a staining pan and as much diluted stain as they can accommodate should be used. If slides are understained or overwashed the true color will be lost, the nuclei and cytoplasm will blend instead of being distinct, and most of the cells will look very fragile. Understained cells look very much like cells stained with acid water.

Refer to Plate IV showing the effects of neutral water, alkaline water, acid water, and excess washing after staining, on blood cells.

Our routine is to neutralize about two liters of water every day. The water should be tested each day since it sometimes becomes acid or alkaline upon standing. Water neutralized in this way may be used with either Giemsa or Wright stain.

Diluting the Stain

Dilute only as much Giemsa stain as desired for the work at hand. While stock Giemsa stain is stable and does not precipitate, diluted Giemsa stain will change within an hour or two.

Measure the neutral distilled water in the flask or cylinder used for the diluted stain. Add 1 drop of Giemsa stain for each 1 c.c. of neutral distilled water, shaking very slightly during the addition of each drop.

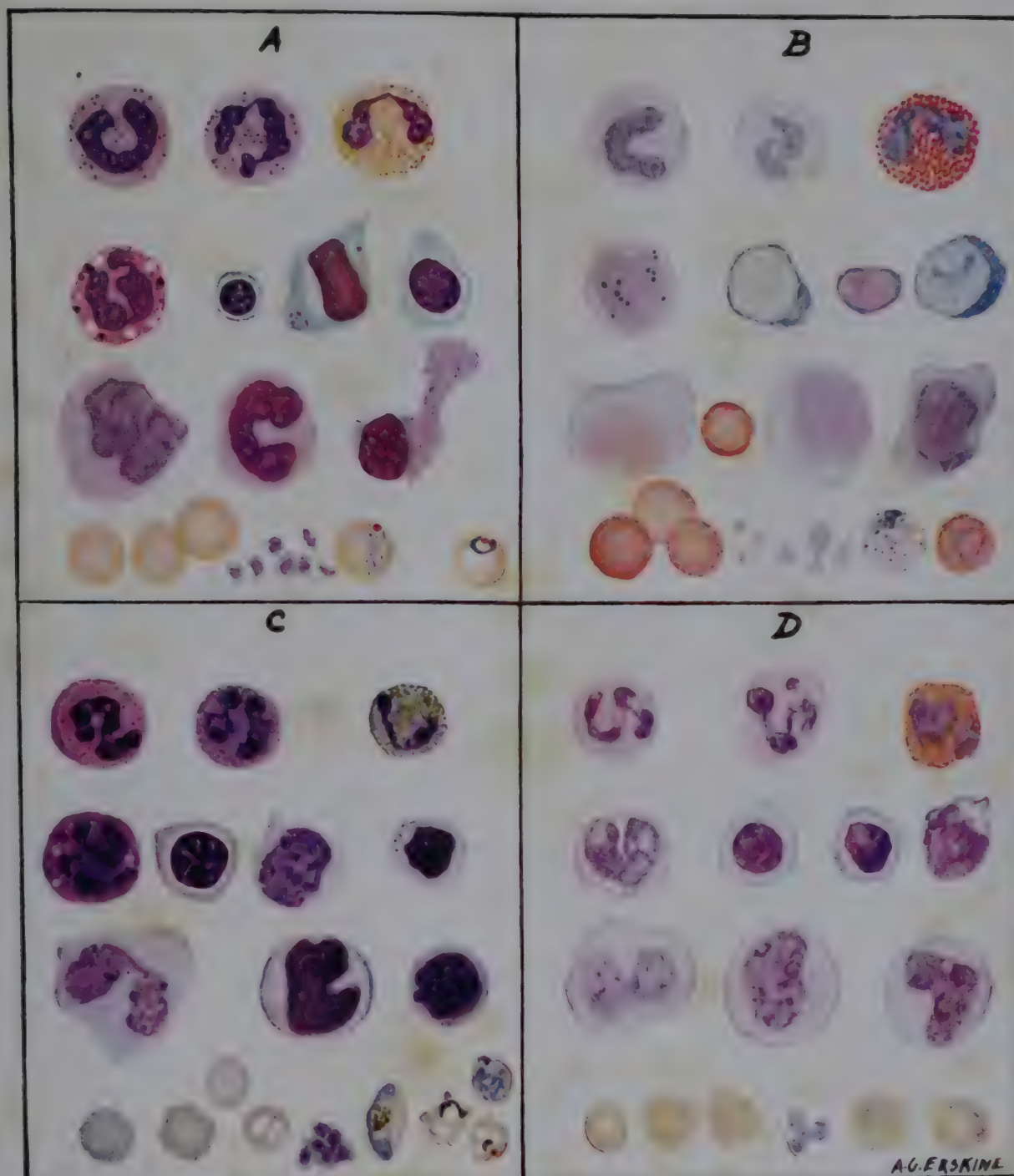
Do not add all the Giemsa stain at once. The matter of shaking after adding each drop is important. Do not shake the flask too vigorously, as a precipitate may form. Shake only enough to insure a good mixture of the stain and the water. The drop should be measured with a dropper the opening of which is not too small.

If a drop bottle is used as a container for Giemsa stain, it must be prepared by allowing neutral distilled water to stand in contact with the glass for at least a week, changing the water each day. The bottle must be dried.

The Giemsa stain (stock) may then be placed in the drop bottle.

Fixing the Film

Absolutely pure, acetone-free methyl alcohol must be used. We recommend the methyl alcohol of the National Aniline and Chemical Company, New York.



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PLATE IV.—BLOOD CELLS AND MALARIAL PARASITES, Giemsa Stain, with

A. Neutral Water
C. Alkaline Water

B. Acid Water
D. Excess Washing after Staining

Top Row: 2 Neutrophiles and an Eosinophile
Second Row: 1 Basophile and 3 Lymphocytes
Third Row: Monocytes
Fourth Row: Erythrocytes, Blood Platelets, and Malarial Parasites



The methyl alcohol should be kept in a Koplin jar, at the end of the day transferred to a bottle, and kept tightly stoppered. Fix the slides by placing them in the jar of methyl alcohol for 3 to 5 minutes. Remove, and allow to dry in the air. The drying of the fixative should always be hastened as much as possible.

Staining the Film

After fixation, place the slides on a staining bridge, and flood them with diluted Giemsa stain. Allow the stain to remain on the slides for 30 minutes, or as long as 45 minutes if desired. Pour off the staining mixture by gently tilting the slide, and wash with neutral distilled water. The washing should not be lengthy, as too long washing will tend to decolorize the stain. Allow to dry in the air. If the slides contain an excess of blue, rewash them after they have dried, then allow them to dry in the air in a vertical position.

Rapid Giemsa Stain No. 1 (W. Ambrose McGee¹).—

Stain the spread for one-half minute with a mixture of one part Giemsa stain to two parts acetone-free methyl alcohol. Mix 20 drops of methyl alcohol and 10 drops of Giemsa stain in a dry test tube, then flood the slide with the entire amount of this mixture for one-half minute. Add an equal volume of neutral distilled water and allow the mixture to remain on the slide for 2 minutes. Flood the slide with neutral distilled water. Dry, and examine. Use only for emergency work.

Rapid Giemsa Method No. 2.—

Take equal parts of stock Giemsa stain and methyl alcohol. Stain one minute. Differentiate until a red color appears. Dry between filter paper.

Changes in Giemsa Staining to Bring Out Special Structures.—

(1) For Malaria.—

To stain blood spreads and thick drop preparations to search for malarial parasites, the water used should be slightly alkaline rather than neutral.

Add 1 drop of a 1% potassium carbonate solution to every 25 c.c. of neutral distilled water before adding the Giemsa stain. One drop of Giemsa stain is added to each c.c. of the water as usual.

(2) For Eosinophiles.—

To stain blood spreads and thick drop preparations to search for eosinophiles, the distilled water used in diluting the Giemsa stain is allowed to remain slightly acid. The granules of the eosinophiles with such staining solution stain decidedly eosin-pink.

(3) For Spirochetes.—

The stain should be alkalized by adding 1 drop of 1% potassium carbonate to each 10 c.c. of distilled water before adding the Giemsa stain.

Fix the slide, after drying, in methyl alcohol for 5 minutes or in ethyl alcohol 20 minutes. Allow to dry.

Flood with diluted Giemsa stain, alkalized, and steam for about 10 minutes, or stain cold for 24 hours.

Wash in running tap water for 3 minutes.

Spirochetes stain red. Other organisms are blue.

Manson Stain, Modified by Schwartz.—

This Manson stain, modified by Schwartz, is used to demonstrate basic punctated cells, as in lead poisoning. The two solutions remain usable for several months and then must be discarded.

Solution I.

Add 2 gm. boric acid and 1 gm. methylene blue to 100 c.c. boiled distilled water, free of carbonic acid, in a Jena flask free of alkali.

¹South. M. J. 25: 484, 1932.

Solution II.

Dissolve 0.28 gm. sodium hydroxide in 100 c.c. boiled distilled water.

Technic.—

Place in a small graduate 6 drops of solution I.

Add 8 drops of solution II.

Shake and dilute to 10 c.c. with boiled distilled water.

Fix blood film in methyl alcohol for 3 to 5 minutes.

Stain with the above mixture for 5 seconds.

Wash, dry, and examine.

Examine with the dark-field.

Erythrocytes are light green-blue.

Basophilic punctation is deep blue to black.

Wright Stain.¹—

No fixative is necessary, as the stain contains methyl alcohol. Neutral distilled water is used. The timing of the stain must be determined when it is manufactured, but usually it is 1 minute staining and 4 minutes after the water has been added.

Flood the slide with Wright stain and allow it to remain in contact with the film for 1 minute. Add neutral distilled water a drop at a time, not too slowly, until there is a mixture of equal parts of distilled water and stain. Allow the mixture to remain for 4 minutes. Flood with neutral distilled water to wash. Dry in the air.

Modified Technic for Staining With Wright Stain (Bercovitz).—

A slight modification of the above technic in making Wright stain has been suggested by Bercovitz.² The apparatus requires two staining jars with covers.

Fill one staining jar with pure methyl alcohol and the other with 30% Wright stain in distilled water.

Immerse the dry blood film in methyl alcohol at least 5 minutes.

Place the slide in the stain for 2 minutes or longer.

Wash and dry as usual.

With this method there is no precipitate on the slide. The stain can be used repeatedly instead of only once. If the fluid levels of the reagents become low, add more solution to bring to the original volume. Do not discard the stain. No changes are noted even when slides are left for as long as an hour in the methyl alcohol. The diluted stain can be used for several months.

May-Grünwald Stain.—

The original communication on blood staining by the May-Grünwald method was published in *Centralblatt für innere Medizin*.^{*} Granulations in the cells of sputum were demonstrated first by tri-acid staining as very fine red spots in the leukocytes.³

It was stated that these granulations were identical with the neutrophilic granulations described by Ehrlich.⁴

May and Grünwald showed some interesting experiments with four kinds of eosin and four kinds of methylene blue directed toward the staining of cells and their granulations. They worked out a mixture of eosin and methylene blue directed toward the specific granulations in blood cells.

Method of preparation:

1 liter of 1% eosin was mixed with 1 liter of 1% medicinal methylene blue and after several days' standing filtered by means of a suction pump. The precipitate was washed with cold distilled water until the filtrate was unstained.

^{*}23: 265-270, 1902.

¹See page 614 for method of making Wright dye and staining solutions.

²Bercovitz, Z.: *J. Lab. & Clin. Med.* 19: No. 4, 1934.

³Virchow Arch. 1899, CLVIII.

⁴In *Centralblatt f. inn. Med.* 1899, No. 30, 1900, No. 14.

The sediment on the filter was allowed to dry. It consisted, as seen under the oil immersion objective, of brown-red needle-form and rod-shaped crystals.

This material was slightly soluble in cold water, more so in warm water, and better in ether, alcohol, acetone, etc., but best in methyl alcohol.

A saturated solution in methyl alcohol was made.

The method of staining is the same as the Wright staining technic.

The May-Grünwald-Giemsa or the Wright-Giemsa Staining of Bone Marrow and Blood Films.—

Spreads of bone marrow and blood may be stained by a combination of May-Grünwald or Wright-Giemsa stain. The advantages of such combined staining are that the nuclear pattern of cells is more distinctive and there is greater regularity of tinctorial reproduction from slide to slide. This combination staining shows the leptochromatic nuclear pattern of young leukocytes exceedingly well. Erythrocytes show a constancy of stain that permits evaluation of hemoglobin content.

Technic.—

Flood slide with Wright stain for 1½ to 2 minutes.

Add neutral distilled water or neutral buffer for 2 minutes.

Pour off and add dilute Giemsa stain for 7 minutes for blood spreads or 10 minutes for bone marrow spreads.

Wash off and dry.

Dilute Giemsa stain is prepared by adding 2 drops of stock Giemsa stain to each c.c. of neutral buffer or neutral distilled water. This diluted Giemsa stain may be kept for 4 to 6 hours, but should not be used beyond this period.

SPECIAL METHODS OF STAINING

Oxydase Staining¹

(To determine the nature of various blood cells.)

Oxydase staining is a very useful method of determining whether or not there is a definite ferment in the blood which gives specific staining to myelogenous, monocytic, and lymphatic elements. A positive reaction to the peroxidase stain is seen in the neutrophilic cells and in the adult monocytes. The method of choice is that advocated by Graham.²

Graham Method

This method is recommended because the stained preparations are permanent.

Solutions.—

Formalin-Alcohol.—

95% Alcohol	90 parts
Formalin (40%)	10 parts

Peroxidase Reagent.—

Alpha-naphthol	1 part
40% Alcohol	95 parts
Hydrogen peroxide	0.2 part

This solution should be not more than one week old.

¹For Oxydase Staining of Tissue see Chapter XIV, Tissue Cutting and Staining.

²Graham: J. M. Res. 35: 1916.

Pyronin Solution.—

Pyronin	0.1 part
40% Alcohol	95 parts
Anilin oil	4 parts
Shake vigorously but do not filter.	

0.5% Aqueous Methylene Blue.—

Methylene blue	0.5 part
Distilled water	100 parts

Filter.

This stain gives better results when diluted 3 to 4 after making the 0.5% solution if National Aniline dye is used.

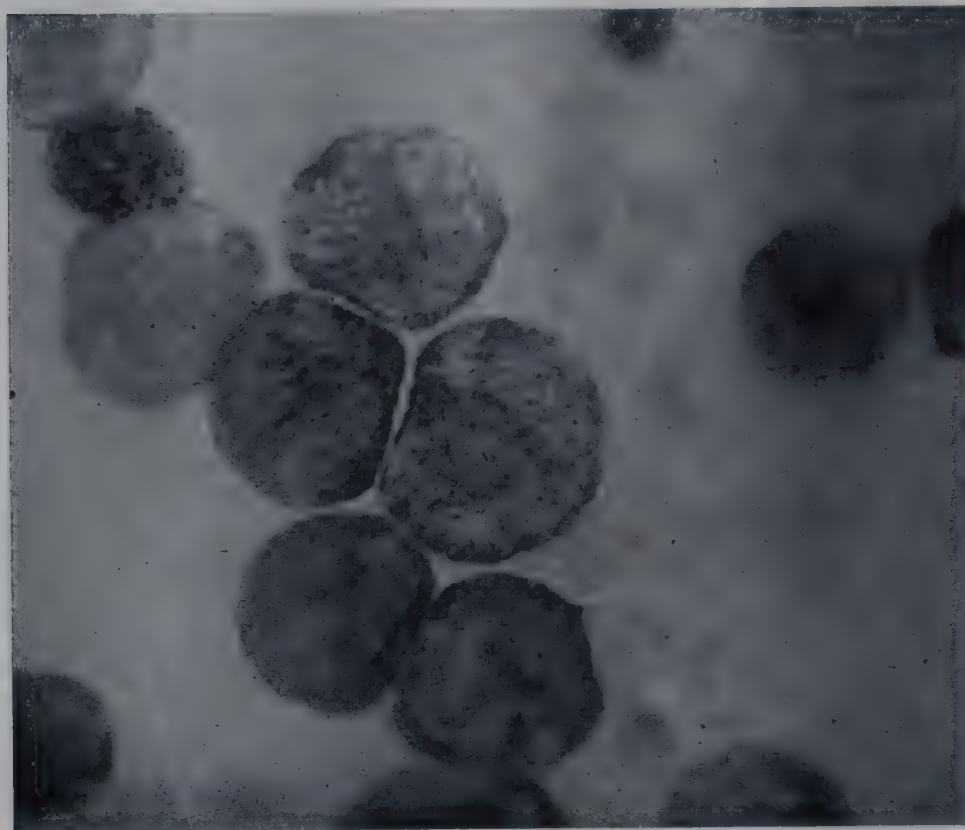


Fig. 167.—Myelocytic leukemia, blood spread, Graham oxydase stain, showing selective staining of granules. ($\times 950$.)

Technic.—

The solutions are kept in Koplins jars.

Make blood films in the usual manner.

After drying in the air, fix in Formalin-alcohol 5 minutes. Rinse thoroughly in running tap water. Drain but do not dry. Dry back of slide.

Place in alpha-naphthol solution (peroxidase reagent) 5 minutes.

Rinse thoroughly in running water. Drain but do not dry. Dry back of slide.

Stain in pyronin solution 10 minutes. Wash. Drain but do not dry.

Stain in methylene blue solution 12 to 20 seconds.

Rinse. Let dry. Examine under low power with the condenser in place to make sure that the staining solutions reacted properly. Look for definite blue stain of nuclei. If not blue enough, stain again in the methylene blue.

Myelocytic elements show red granules. *Lymphocytic* elements stain blue. All nuclei stain blue. *Monocytes* are not characteristically stained, but the "pink dust" stains red, and appears as very fine stippling.

Before staining the patient's blood film, it is best to stain a film of freshly prepared normal blood to determine timing of stains and staining characteristics. The staining solutions seem to give better results if they are allowed to stand for 24 hours before using, although they may be used immediately after manufacture if there is any emergency.

Oxydase Reaction According to the Methods of Sato and Sekiya

Reagents.—

1. Copper sulphate 0.5 gm.
Distilled water 100.0 c.c.
2. Benzidine solution. Moisten 0.02 gm. benzidine, special for blood, with a few drops of water. Then grind. Add 200 c.c. distilled water and filter. To filtrate add 0.25 c.c. of 3% hydrogen peroxide. Keep this solution in a brown bottle in the dark when not in use. It will remain good for a year although some precipitation occurs.
3. Safranin, 1% aqueous solution.

Technic.—

To a fresh, dry blood film apply the copper sulphate solution for 20 seconds. Drain off and apply the benzidine solution for 8 minutes. Drain this off and add the safranin solution for 2 minutes. Wash and dry. Examine under oil immersion.

The peroxidase granules of myelocytes and segmented neutrophils have a deep green-blue color while the nuclei are stained orange with the counter-stain. Granules vary in number and size. Eosinophils show large heavy granules while others are densely granular. Myelocytes show only a few granules; the younger they are, that is, the nearer in age to the myeloblast, the fewer granules they have. The lymphocytes have no granules and the monocytes only a few near the nucleus. This reaction magnifies granules and serves to distinguish between the white cells of bone marrow origin and those of lymphocytic or reticuloendothelial origin.

To Count Oxydase Stained Spreads of Blood.—

Use the four-field meander method of counting (page 626). Count 200 cells, 50 in each field. Record these simply as oxydase-positive (myelocytic) elements, oxydase-negative (lymphocytic) elements, and oxydase-partially-positive (monocytic) elements. Report the percentage of each type of cell. For example:

		Normal
Myelocytic elements (oxydase-positive) -----	85%	(63 to 77%)
Lymphocytic elements (oxydase-negative) -----	13%	(21 to 30%)
Monocytic elements (oxydase-partially-positive) ---	2%	(4 to 8%)

In the above report, if this is leukemia, obviously it is myelocytic. The normal white cells of the differential count, when oxydase-stained, occur in the following numbers: oxydase-positive, 63-77%; oxydase-negative, 21-30%; oxydase-partially-positive, 4-8%. Myeloblasts, promyelocytes, all myelocytes, juveniles, stabs, segmented neutrophils, eosinophils, and basophils stain oxydase-positive except in unusual cases when they do not carry the oxydase ferment. Lymphoblasts, prolymphocytes, large and small lymphocytes, plasma cells, all carry no ferment and therefore are oxydase-negative. Monoblasts, promonocytes, and mature monocytes usually have a few, extremely small, oxydase-positive granules.

Demonstration of Siderocytes or Sideroblasts¹

Siderocytes and sideroblasts are described on page 631.

Material Required.—

Spreads of Blood or Bone Marrow.—

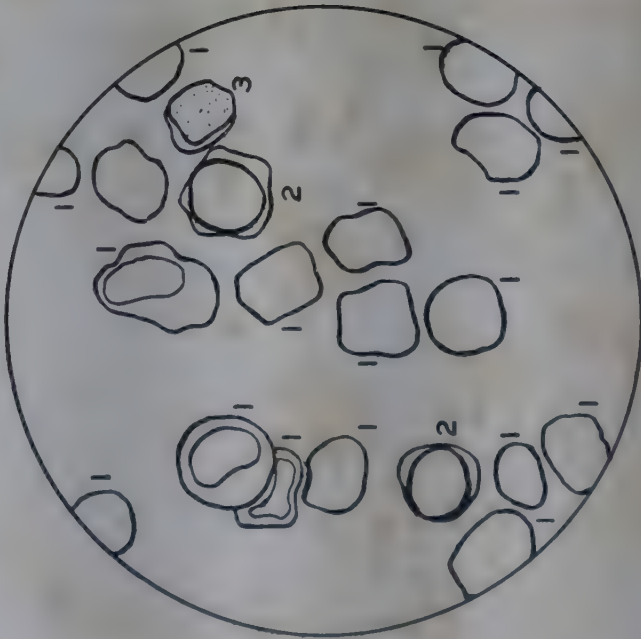
Formalin.

2% Aqueous Solution of Potassium Ferrocyanide.—

2 gm. of potassium ferrocyanide per 100 c.c. of distilled water. This should be aged for several days.

¹Kaplan, E., Zuelzer, W. W., and Mouriquand, C.: Blood 9: 203, 1954.

GRAHAM OXYDASE STAIN



MYELOCYTIC LEUKEMIA

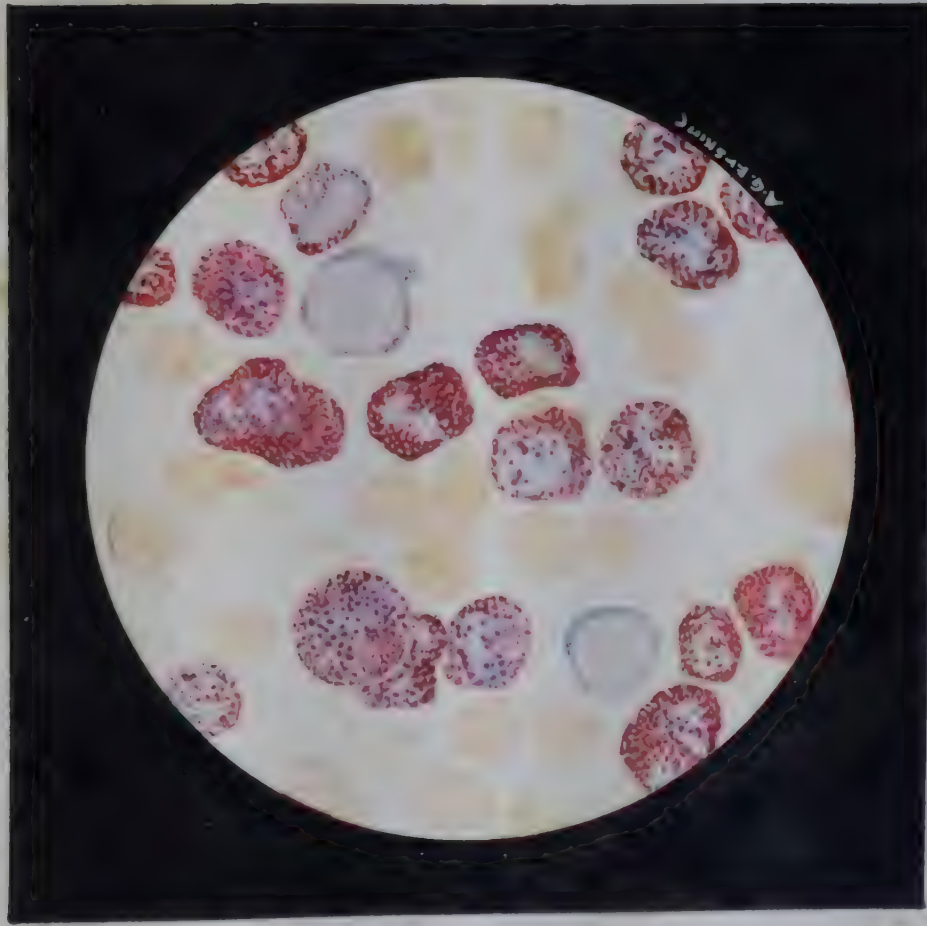
- 1. Myelocytic cells
- 2. Lymphocytic cells
- 3. Monocytic cells



LYMPHOCYTIC LEUKEMIA

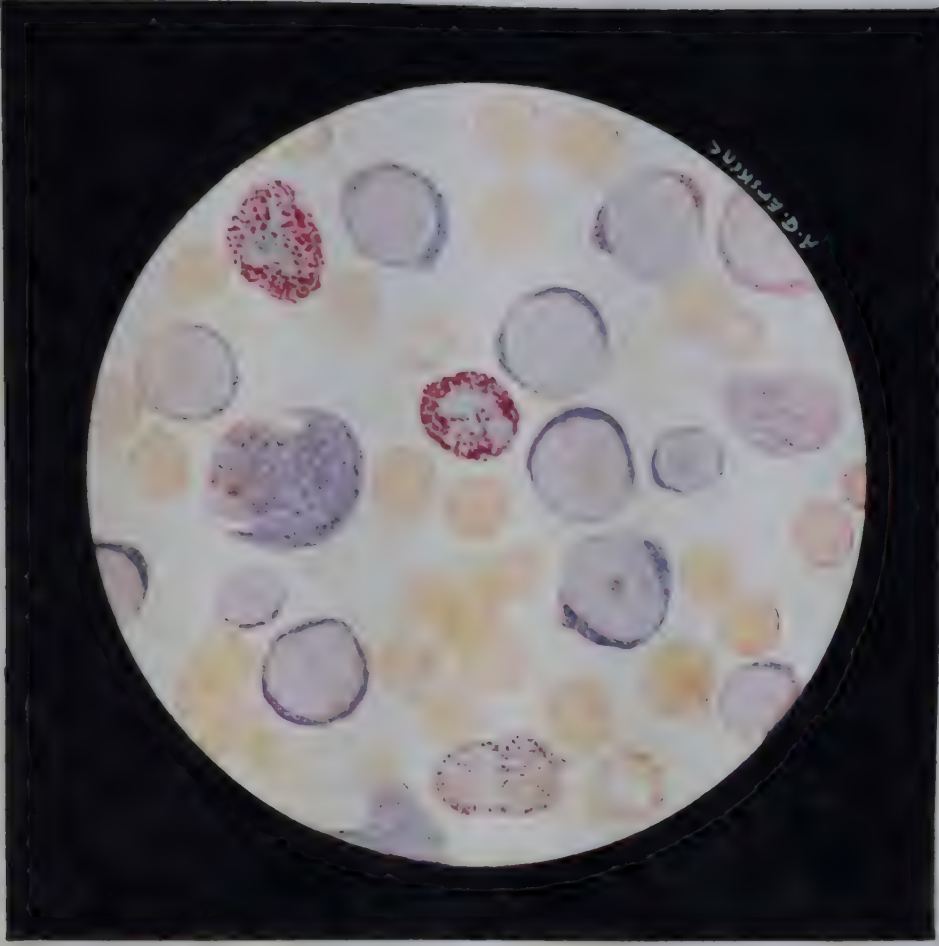
- 1. Lymphocytic cells
- 2. Monocytic cells
- 3. Myelocytic cells
- 4. Nuclear fragments

GRAHAM OXYDASE STAIN



MYELOCYTIC LEUKEMIA

X 950



LYMPHOCYTIC LEUKEMIA

X 950

PLATE V.



2% Hydrochloric Acid.—

2 c.c. of concentrated hydrochloric acid added to distilled water and diluted to 100 c.c. with distilled water.

Stock Basic Fuchsin Solution.—

Dissolve 1 gm. basic fuchsin

in 10 c.c. absolute alcohol.

Add .90 c.c. of 5% aqueous phenol.

Working Counterstain.—

Add 6 c.c. of the stock basic fuchsin solution to 100 c.c. of distilled water.

Filter all solutions just prior to use.

Technic.—

Place blood or bone marrow films in a Koplín jar and cover the top of the jar with a piece of absorbent paper saturated with Formalin.

Allow the slides to remain in this Formalin vapor atmosphere for 30 minutes.

Stain 1 hour in a mixture of equal parts of ferrocyanide and hydrochloric acid solutions.

Wash in tap water.

Counterstain with dilute basic fuchsin for 30 to 60 seconds.

Remove excess basic fuchsin by washing in tap water and dipping in absolute ethyl alcohol.

Air dry.

Examine under oil immersion.

Siderocytes and sideroblasts will contain iron in the form of blue-staining granules within the cytoplasm.

DIFFERENTIAL COUNT

The differential count is also known as the qualitative leukocyte count. Its purpose is to determine the proportion of leukocytes of different types. This was done first in films by Einhorn¹ in 1884, followed by Kronig² in 1899, on spinal fluid, Widal and Ravaut³ in 1900 (also on spinal fluid); by Simpson⁴ in 1906; Sheaff⁵ in 1912; Schilling⁶ in 1913; Nieuwenhuijse⁷ in 1914 and Johns⁸ in 1914. Up to the announcement of Schilling in 1913, the method was known as the "Ehrlich" count for the reason that the types of cells described and counted were those noted by Ehrlich in his classical studies on the tinctorial qualities of various blood cells. It was Arneth who attempted to show that we might divide the immature cells into various forms and thus secure much more information as to the severity of infection present. His studies and suggestions, while essentially important, were not adopted because of the intricate details necessary to make a so-called Arneth count. Furthermore, according to Arneth's theory, leukocytes leave the bone marrow as young forms with simple indented nuclei and these cells become changed into mature polymorphonuclear leukocytes in the circulating blood. Schilling denies that the presence of the increased percentage of immature cell forms in the blood

¹Einhorn, M.: Inaugural-Dissertation, Berlin, 21, March, 1884.

²Kronig, G.: Verhandl. des Congr. f. inn. Med., 11-14, 17: 569, 1899.

³Widal, F., and Ravaut, P.: Compt. rend. Soc. de biol. 52: 648, 651, 653, June 30, 1900; and 52: 838, Oct. 13, 1900.

⁴Simpson, C.: J. Michigan M. Soc. 5: 367, July, 1906.

⁵Sheaff, P. A.: J. A. M. A. 58: 1941, June 22, 1912.

⁶Schilling, V.: Deutsch. med. Wchnschr. 39: 1985, Oct. 9, 1913.

⁷Nieuwenhuijse, P.: Nederl. tijdschr. geneesk. 58: 872, March 21, 1914.

⁸Johns, F. M.: New Orleans M. & S. J. 67: 332, Oct., 1914.

necessarily means an increased production of leukocytes by the bone marrow. He believes that the picture may be changed by immature cells, which enter the blood, being injured by bacterial or other toxins so that they fail to undergo differentiation. For instance, in typhoid fever, with a decrease in the total number of white blood cells in the blood and increase in the number of immature neutrophils, Schilling explains this as being due, not to excessive destruction of white cells in the circulation with regenerative overactivity of the bone marrow, but rather to actual depression of marrow function with a discharge into the blood of injured young neutrophils which do not become differentiated into the more segmented bodies. While Schilling's work was first published in 1913 it seems to have escaped general attention, at least in the United States and practically so on the Continent, until a translation of his classic book from the German was published in 1929.* His remarkably simple classification of immature leukocytes, together with an interpretative formula, has been followed by a rather wholesale departure from the other methods of differential blood counting so long in vogue. Since the popularization of the Schilling method, other methods of counting have been introduced, notably that by Cooke and Ponder, in which five different types of cells are classified, dependent upon the number of nuclear lobes. Any increase in the number of cells of the first two forms, that is, the youngest type, is interpreted as a shift to the left. Then again we find the simplified Arneth count of Ponder and Krumbhaar, in which neutrophils are divided into three classes. Finally, Farley and others have offered the filament and nonfilament neutrophile count as a method of choice in the diagnosis and prognosis of infection.

We do not find, however, that any count other than the Schilling gives as complete an understanding of the effects of infection in regard to diagnosis and prognosis.

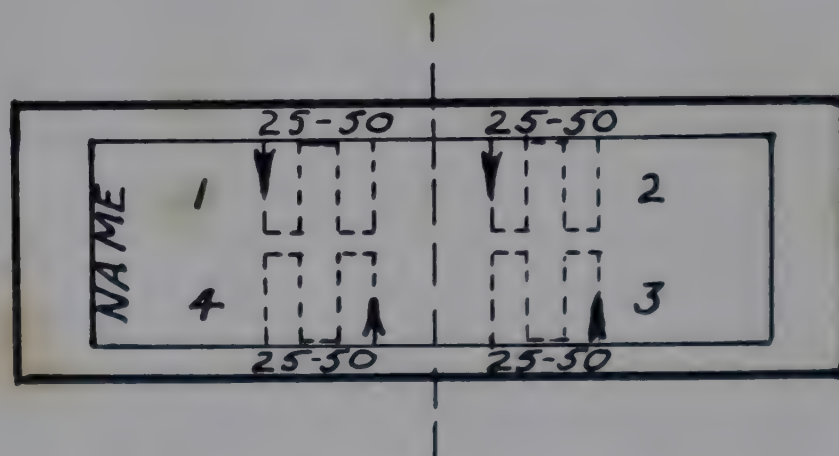


Fig. 168.—The "four-field meander" method of differential counting.

Tabulation of the Differential Count

The leukocytic blood picture is obtained by the differential count, which consists in counting 100, 200, or 500 stained leukocytic cells, arranging them in the classification to which they belong, and reporting the percentage of each cell tabulated.

The "four-field meander" method is recommended. Place a drop of oil in the middle third of each half of the film, as shown in Fig. 168. Twenty-five

*Schilling, Victor C.: *Das Blut Bild*. G. Fischer, translated by R. B. H. Gradwohl. St. Louis, 1929, The C. V. Mosby Co.

or 50 cells are usually counted in each field. The count is made by beginning the enumeration of the cells at the edge of the film, working toward the center (by moving the mechanical stage), going not quite to the center, then working back to the edge, then toward the center until the desired number (25 or 50) of cells has been tabulated. After counting either 25 or 50 cells, as the



Fig. 169.—Marbel calculator for tabulating differential counts.

Normal %	10	20	30	40	50	60	70	80	90	100	10	20	30	40	50	60	70	80	90	200 Cells Counted	%
0.5 (0.1) Baso- phile																1				1	1/2
3 (2.4) Eosino- phile		/						/					/			1	1			5	2 1/2
0 Myelo- cytes																					
0.5 (0.1) Juvenile				/																1	1/2
4 (3.5) Stabs	/	/		/				//	/			/				1	1		/	10	5
63 (58-66) Seg- ments	/		//	//	///		//	//		/	//		//	///				///	///	123	61 1/2
23 (21-30) Lympho- cytes	///	//	///	/		///	///	/	///	///	//	//	///	///	/	///	///	/	///	49	24 1/2
6 (4.8) Mono- cytes		/						//		//	/	//				1	1		/	11	5 1/2
																				200	100

REMARKS:
White cells: 12,000
Atypical cells (describe):
Hematocrit (cell volume %):
Mean corpuscular volume:
Mean corpuscular hemoglobin:
Mean corpuscular hemoglobin concentration:

Red cells: 4,500,000
Hemoglobin: 13.26 gm. per 100 c.c. Spencer, 84.9%
Polychromasia: +
Normoblasts:
Megakaryoblasts:
Anisocytosis: slight
Special forms:
Parasites:

Color index:
Volume index:
Saturation index:
Basophilic punctation:
Poikilocytosis:
Platelets:

Fig. 170.—Hemogram chart including Schilling classification.

case may be, shift to the next indicated field, and begin the enumeration there. Continue in this manner until either 100 or 200 cells have been counted. It is our custom to count 50 cells in each field, and divide the total of each cell counted by 2 to get the percentage of that type of cell.

Do not count only the edges since the large cells tend to accumulate there; and since the lymphocytes congregate in the center, an inaccurate count is

obtained if only the center is counted. By using the "four-field meander" method of counting a more accurate count is obtained.

Now construct a "hemogram" according to Schilling (page 781) and record the percentage of each cell counted. Record any abnormalities of the erythrocytes, the presence of blood parasites, or unusual staining, such as toxic granules. All reports on the examination of differential counts must include an enumeration of abnormalities encountered in the erythrocytic cells. When the differential count is completed, a survey examination of the erythrocytes should be made so as not to overlook any pathologic findings.

There are various methods of mechanically computing the different cells in the differential count. The Marbel Calculator is one type of instrument, Fig. 169.

Record of Erythrocytic Cells in a Differential Count

In a differential count, all changes in erythrocytes are noted and a written report made of the findings. Observe first for anisocytosis, then poikilocytosis, polychromasia, basophilic punctation, then for any other abnormalities of the cells. The actual numbers of all types of nucleated red cells must be given per 100 leukocytes, together with a record of karyorrhexis and mitosis. Report anisocytosis, poikilocytosis, polychromasia, and basophilic punctation as marked, moderate, or slight. Report in per cent the number of the various types of nucleated reds (polychromatic megaloblasts, orthochromatic megaloblasts, etc.) per 100 leukocytes on the slide. If Howell-Jolly bodies, Cabot ring bodies, or any other changes are noted in the erythrocytes, such findings must be recorded in the written report which is presented to the physician. In addition to the degree of basophilic punctation, it is well to report the size of the dots as fine, medium, or coarse.

The erythrocytes are classified according to the size of the majority of erythrocytes and their pigment content into seven groups as follows:

1. Normocytic normochromic, normal-sized erythrocytes with normal pigment content.
2. Normocytic hypochromic, normal-sized erythrocytes with decreased pigment content.
3. Microcytic hypochromic, small erythrocytes with decreased pigment content.
4. Microcytic normochromic, small erythrocytes with normal pigment content.
5. Macrocytic normochromic, large erythrocytes with normal pigment content.
6. Macrocytic hypochromic, large erythrocytes with decreased pigment content.
7. Macrocytic "hyperchromic," large erythrocytes with apparent increased pigment content. This is often called macrocytic normochromic.

If the majority of erythrocytes are normal in size, but there are scattered macrocytes, this would be reported as normocytic with a tendency to enlargement of the cells.

DESCRIPTION OF STAINED CELLS

Erythrocytes

1. **Normocytes or Erythrocytes.**—Erythrocytes, or red blood cells, are found normally in the circulating blood. They are biconcave disks without nuclei, 7.5 to 8.0 μ in diameter and 1.7 to 2.2 μ thick. Normal red blood cells are orthochromatic. The orthochromatic cells are the mature cells. Polychromatic cells are younger cells, and may be found in the proportion of 2 to 10 faintly polychromatic cells per 1,000 orthochromatic erythrocytes, in normal blood films. They stain a uniform dull orange when orthochromatic, or blue-gray when polychromatic. Any variation from this type of cell is pathologic or the result of poor technic.

2. **Macrocytes.**—Macrocytes are erythrocytes that are larger than the normal cells, ranging up to 14 μ in diameter. They contain no nuclei. They are not found normally in the circulating blood. They may be polychromatic or orthochromatic.

3. **Megalocytes.**—Megalocytes are giant erythrocytic cells (15-18 or 20 μ in diameter), usually oval, never found normally in the circulating blood. They have no nuclei.

4. **Microcytes.**—Microcytes are very small red blood corpuscles (5 μ in diameter), not found normally in the circulating blood. True microcytes are usually hypochromic.

5. **Erythrogonia.**—The erythrogonia is considered by Schilling to be the parent cell of the erythrocytes. These cells are not found normally in the circulating blood. The cytoplasm is entirely free from hemoglobin. It stains a deep blue. The nucleus is round, and deep purple. There are no granules. This cell can be identified only in connection with the finding of normoblasts and erythroblasts in the erythropoietic centers, or during blood crises. These cells are usually larger than megaloblasts. The nuclei usually show nucleoli. These cells resemble megaloblasts except that the cytoplasm is a much deeper blue, there is only a narrow rim of cytoplasm, and the nuclei contain nucleoli.

6. **Megaloblasts.**—Megaloblasts are never found in the circulating blood in health. They are remarkably large (giant) nucleated erythrocytes. The cell is slightly oval. The cytoplasm contains hemoglobin in varying quantities. The cells, therefore, may be polychromatic or orthochromatic; that is, they stain a deep gray-blue, a bluish orange, or the orange of the mature erythrocyte. The cytoplasm of orthochromatic megaloblasts is markedly hyperchromatic. The nucleus is oval or round with a fine chromatin structure. If the cell is in the process of mitosis, the nucleus has the typical mitotic appearance. It stains a deep purple. There are no granules. The nucleus always has a very fine chromatin structure.

7. **Macroblasts.**—Macroblasts are nucleated red blood cells larger than the normal erythrocytes, but smaller than the megaloblasts. They are never found in the blood stream in health. They are found in conditions of erythrocytic hyperregeneration and in injury to the bone marrow. The cytoplasm may be either orthochromatic or polychromatic. The nucleus is round, and has a coarse chromatin structure. It stains deep purple. There are no granules. The nucleus is frequently excentrically situated,

8. **Normoblasts.**—Normoblasts are nucleated red blood cells the same size as normal erythrocytes. They are never found normally in the circulating blood. The cytoplasm may be orthochromatic or polychromatic. The nucleus is deep purple, and contains a structure that resembles the spokes of a wheel. If the cell is overstained the entire nucleus stains a uniform deep purple. There are no granules. Giant normoblasts are sometimes found. They are as large as megaloblasts with small nuclei that characterize the normoblasts. Do not confuse normoblasts and lymphocytes. The polychromatic normoblast has an opaque blue or gray cytoplasm. The lymphocyte has deep blue grainy cytoplasm, with an unstained perinuclear zone usually visible. Nuclei of normoblasts are much darker than nuclei of lymphocytes, and except under conditions of degeneration they are always spherical.

9. **Microblasts.**—Microblasts are small nucleated erythrocytes which may be either orthochromatic or polychromatic. Do not confuse them with lymphocytes. The nuclear structure will make the differentiation. They are never found normally in the circulating blood.

10. **Polychromatic Erythrocytes (Basophilic Erythrocytes).**—Polychromatic erythrocytes are red blood cells which do not stain uniformly orange, but show bluish coloration. They may or may not show stippling. Those which show stippling are called *punctated* or *stippled* erythrocytes. The term “basophilic punctation” is used to describe basophilic stippled erythrocytes. Stippling appears as fine or coarse dots stained blue or purple scattered either around the edge or throughout the entire red cell. A blood picture in which the number of polychromatic erythrocytes is increased is described as “polychromasia,” or “diffuse basophilia,” or a “polychromatic blood picture.” One or two polychromatic erythrocytes may be found to every five hundred normal orthochromatic cells, normally.

Polychromasia and stippling were formerly regarded as signs of degeneration in the red cell, stippling being commonly referred to as “granular degeneration.” With the discovery of the reticulocyte in supravital stained preparations, the question arose as to whether or not abnormal staining of this type was a sign of youth or a sign of degeneration. Hawes¹ produced evidence that the polychromatic cell of a Leishman-stained film was related to the reticulocyte of a vitally stained preparation and suggested that the stippled cell was merely a variant of the polychrome. Key,² in 1924, confirmed the work of Hawes in relation to the polychromatic cell, and pointed out that the supravital method of staining is more sensitive in detecting reticular material than is Leishman’s stain for polychromasia. There is, of course, considerable doubt as to whether or not the stippled cell is in any way related to the reticulocyte. Piney³ suggested that stippling may be nothing more than nuclear remnants. Samson Wright,⁴ in 1931, although believing that stippling is of cytoplasmic origin, did not connect it with the polychromatic cell. Maximow and Bloom⁵ state that “it is possible that the stippled condition is a mani-

¹Hawes, J. B.: Boston M. & S. J. citi: 493, 1909.

²Key, J. A.: Arch. Int. Med. 28: 511, 1921; Am. J. Physiol. 70: 86, 1924.

³Piney, A.: Diseases of Blood, ed. 1, London, 1932, p. 2.

⁴Wright, Samson: Applied Physiology, ed. 3, London, 1931, p. 205.

⁵Maximow, A. A., and Bloom, W.: Text-book of Histology, ed. 1, Philadelphia, 1930, p. 60.

festation of immaturity much the same as polychromasia." In this connection, Britton¹ carried out an interesting series of experiments to confirm the relation of the polychromatic cell to the reticulocyte, to establish the meaning of the phenomenon of stippling, and to determine the relation between the stippled cell and the reticulocyte and polychromatic cell. His experiments were designed to provide accurate observations on the numbers of reticulocytes, polychromatic cells, and stippled cells produced by: (1) phenylhydrazine, which results in abundant polychromasia; (2) colloidal lead, which results in abundant stippling; and (3) the superposition of colloidal lead poisoning upon previously produced phenylhydrazine poisoning.

Work was done on healthy adult rabbits, which were injected intravenously with 0.44 per cent colloidal lead and with a 4 per cent aqueous solution of phenylhydrazine hydrochloride rendered neutral to phenolphthalein with sodium hydroxide. Reticulocyte counts were made with supravital staining and counts of polychromasia and stippling were made by determining the number of these cells in proportion to red cells which were visible in an ordinary dried Leishman-stained blood film. Large doses of the drug were used in order to produce an acute poisoning. It has been shown that repeated small doses are almost without action, and that the chronic lead poisoning of human beings is almost impossible to produce in the rabbit.

This experimental work showed that reticulocytes produced by poisoning either with phenylhydrazine or with lead are, so far as can be seen from ordinary staining, identical with normal reticulocytes. The number of polychromatic and stippled cells is paralleled by the number of reticulocytes. It seemed, according to this investigator's results, that the polychromatic material, stippling, and reticulum are the same. He obtained sufficient evidence that polychromasia and stippling are both identical with reticulation. He concluded that stippling is the same chromatic substance slightly altered by lead or other poison as the polychromatic characteristics of red cells in general.

11. Reticulocytes.—Reticulocytes are young polychromatic erythrocytes which show a "reticulum" or net structure when stained with supravital methods. (See 10 above.) These nets look like fine lines throughout the cell. Two to ten reticulocytes to every thousand normocytes is a normal finding.

12. Siderocytes.—"Siderocyte" is a term introduced by Gruneberg² to refer to red blood cells which contain nonhemoglobin iron demonstrable by the Prussian blue reaction. The iron appears in the form of blue-staining granules within the red cell and has been demonstrated in normal blood especially after splenectomy.^{3, 4} See page 623 for method of staining.

Recently Kaplan and associates⁵ have studied the presence of **sideroblasts** (nucleated erythrocytes giving a positive Prussian blue reaction) in the bone marrow. They found them ranging from 20 to 90 per cent in normal subjects and in patients suffering from a variety of anemias. The outstanding excep-

¹Britton, C. J. C.: *Lancet* 1: 1173-1176, June 3, 1933.

²Gruneberg, H.: *Nature* 148: 114, 1941.

³Boniach, I., Gruneberg, H., and Pearson, J. E. G.: *J. Path. & Bact.* 55: 23, 1943.

⁴Pappenheimer, A. M., Thompson, W. P., Parker, D. D., and Smith, K. E.: *Quart. J. Med.* 14-15: 75, 1945.

⁵Kaplan, E., Zuelzer, W. W., and Mouriquand, C.: *Blood* 9: 103, 1954.

PLATE VI.—BLOOD CELLS—GIEMSA STAIN.

- (1) Myeloblast.
- (2) Promyelocyte.
- (3) Neutrophilic myelocyte (form usually seen in myelocytic leukemia).
- (4) Neutrophilic myelocyte (form usually seen in infections).
- (5) Eosinophilic myelocyte.
- (6) Basophilic myelocyte.
- (7) Juvenile (neutrophilic).
- (8) "Stab" (neutrophilic).
- (9) Twisted "stab" (neutrophilic).
- (10) Degenerated "stab" (neutrophilic).
- (11) Segmented neutrophile.
- (12) Eosinophile.
- (13) Basophile.
- (14) Lymphoblast.
- (15) Large lymphocyte.
- (16) Small lymphocyte.
- (17) Reticuloendothelial cell.
- (18) Monocyte (formerly called "transitional").
- (19) Monocyte.
- (20) Monocyte.
- (21) Endothelial element.
- (22) Atypical promyelocyte.
- (23) Micromyeloblast.
- (24) Myeloblast with two nuclei, no mitosis.
- (25) Plasma cell.
- (26) Large plasma cell.
- (27) Polychromatic megaloblast.
- (28) Orthochromatic macroblast.
- (29) Orthochromatic normoblast.
- (30) Orthochromatic microblast.
- (31) Normoblast with nucleus showing karyorrhexis.
- (32) Hyperchromic megalocyte.
- (33) Macrocyte.
- (34) Normocyte.
- (35) Microcyte.
- (32 to 35) Example of anisocytosis.
- (36) Erythroblast with mitotic nucleus.
- (37) Faintly polychromatic erythrocyte.
- (38) Polychromatic erythrocyte.
- (39) Basophilic punctation, fine.
- (40) Basophilic punctation, coarse.
- (41) Poikilocytes.
- (42) Marginal granule.
- (43) Ring form of malarial parasite.
- (44 and 45) Cabot ring bodies.
- (46) Erythrocyte showing hypochromia.
- (47) Hyperchromic erythrocyte.
- (48) Blood platelets.

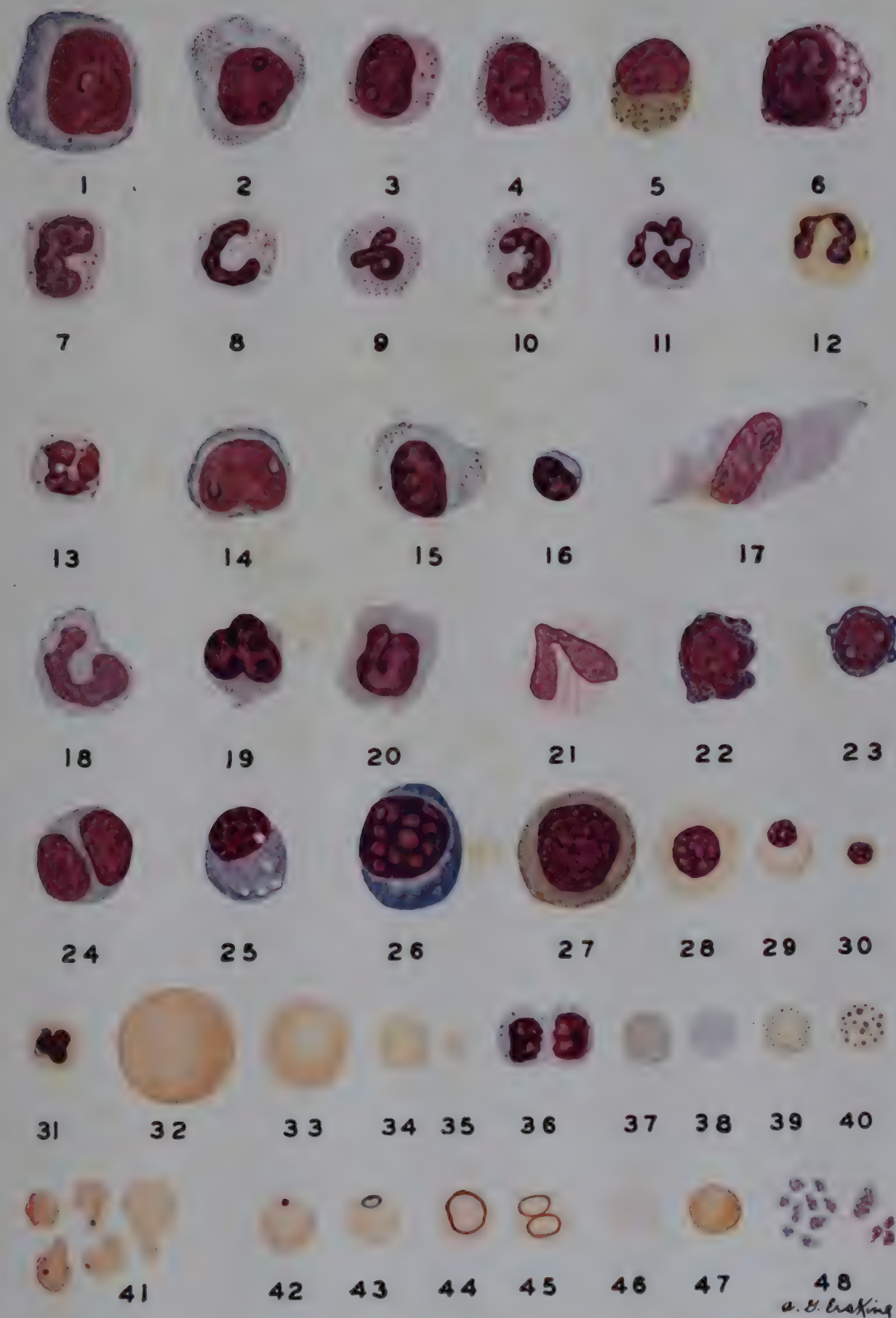


PLATE VI.—BLOOD CELLS—GIEMSA STAIN.

(See explanation of figures on opposite page.)

tion was a group of 55 infants and children with iron deficiency anemia. In these hypochromic anemias, there was a marked reduction in sideroblasts ranging from 0.5 to 1.5 per cent, but usually below 1 per cent. With intravenous iron therapy, sideroblasts returned to normal values promptly.

The method of demonstrating siderocytes is given on page 623.

13. **Spherocytes.**—Spherocytes are rounded red blood cells with a diameter of approximately 4 microns. In blood spreads, they appear circular and seem to have a much darker stain than the normal cells; this is due to an increased thickness. They occur most frequently in congenital hemolytic icterus, but may be present in occasional cases of acquired spherocytosis.

Abnormalities of Erythrocytes

14. **Karyorrhexis.**—Karyorrhexis of the immature erythrocytes appears as bizarre clover-leaf nuclear shapes. This is indicative of beginning disintegration of the nucleus, and is, of course, a pathologic finding. *Karyolysis* is solution of the nucleus of immature erythrocytes.

15. **Nuclear Spheres.**—Nuclear spheres of erythrocytes are distinctly rounded droplike liquid remnants of nuclei of all sizes. They stain very dark purple. They are pathologic.

16. **Erythrocytic Shadows.**—Erythrocytic shadows are dehemoglobinized erythrocytes which occur while making the preparation. They are caused by lack of resistance of the erythrocytes. They stain pale pink.

17. **Erythrocytic Crescent.**—Crescent bodies are delicate, almost colorless, veil-like disks, 40 to 50 microns in size, with pink crescents in the margin. They might be mistaken for parasites, but are nonspecific general indicators of anemia. They often are the result of faulty technic.

18. **Schüffner Punctuation.**—Schüffner punctuation is a reddish violet stippling of erythrocytes occurring in tertian malaria. They are scattered over the whole erythrocyte, occasionally appearing as distinctly connected by a reticular structure.

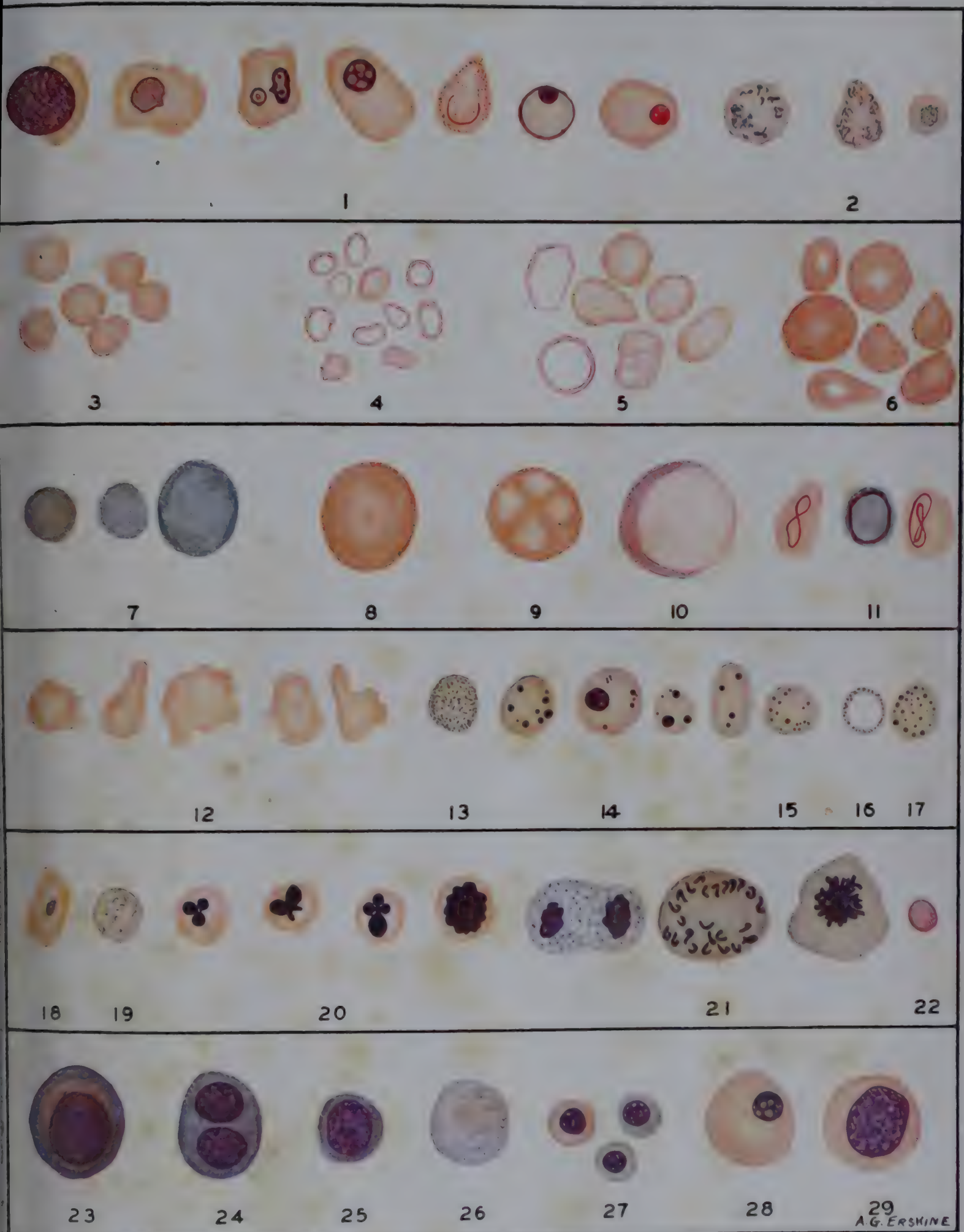
19. **Pernicious Maculation.**—Pernicious maculation of the erythrocytes occurs as a coarse dark violet maculation. This is seen in severe forms of falciparum malaria.

20. **Marginal Corpuscles.**—Marginal corpuscles are very small (less than one-quarter of a micron) single or double granules in an erythrocyte, in an extremely excentric or peripheral position. They are very easily detachable, are seen in only a few erythrocytes in the film, stain red, and are seen particularly in the regenerative anemias.

21. **Inner Bodies.**—Inner bodies are bodies in erythrocytes demonstrated with the supravital staining methods. They include *Ehrlich's hemoglobinemic inner bodies*, which are hemoglobin-containing, vividly eosin-colored, spherical bodies, easily expelled, forming a part of the *schistocytes* (see page 636). They occur only in severe, toxic anemias. *Heinz's blue granules* are evidently identical with Ehrlich's inner bodies. *Schmauch's inner bodies* in cats are partially identical with Ehrlich's inner bodies, and partially with nuclear spheres (see above).

PLATE VII. VARIATIONS IN ERYTHROCYTIC CELLS

1. Ferrata's views on the formation of Cabot ring bodies.
2. Reticulocytes—supravital stain.
3. Normochromic normocytic cells.
4. Hypochromic microcytic cells.
5. Hypochromic macrocytic cells.
6. "Hyperchromic" macrocytic cells.
7. Polychromatic erythrocytes.
8. Orthochromatic megalocytes—hyperchromic.
9. Orthochromatic megalocyte showing endoglobular degeneration.
10. Erythrocytic crescent.
11. Cabot ring bodies.
12. Poikilocytes.
13. Basophilic punctation, medium.
14. Howell-Jolly bodies, or nuclear spheres, some in a nucleated red cell.
15. Basophilic punctation in a cell with a marginal granule.
16. Basophilic punctation, fine.
17. Basophilic punctation, coarse.
18. Blood platelet on an erythrocyte.
19. Supravital stain of an erythrocyte, showing reticulum and a hemoglobinemic inner body.
20. Nucleated erythrocytes showing karyorrhexis.
21. Erythroblasts in mitosis.
22. Karyogenic metachromasia.
23. Polychromatic megaloblast.
24. Polychromatic megaloblast with two nuclei, without evidence of mitosis.
25. Polychromatic macroblast.
26. Megalocyte with erythrocytes.
27. Normoblasts, one of which is orthochromatic, and two polychromatic.
28. Giant normoblast.
29. Orthochromatic megaloblast.



VARIATIONS IN ERYTHROCYTIC CELLS

PLATE VII.

29
A.G. ERSKINE

22. Howell-Jolly Bodies.—Howell-Jolly bodies are peculiar spherical nuclear remnants seen in erythrocytes. They may graduate down to the size of a needle point. They are present after splenectomy and in severe anemias. They stain deep purple.

23. Karyogenic Metachromasia (Ferrata).—Karyogenic metachromasia is a faintly wine red discoloration of erythrocytes due to the presence of fully dissolved chromatin particles. It is not found normally.

24. Cabot Ring Bodies.—Cabot ring bodies are very delicate, circular, figure-of-eight or loop-shaped, threadlike bodies, frequently seen in polychromatic or punctated erythrocytes, sometimes seen in erythrocytes with nuclear spheres. They are seen in very severe anemias. Cabot¹ described these bodies as red and blue staining circular and twisted bodies within basophilic, polychromatic, and, infrequently, orthochromatic erythrocytes. They were brought to his attention by one of his associates in a case of lead poisoning and thereafter observed by him in the blood of patients suffering from pernicious anemia, lead poisoning, and lymphocytic leukemia. There has been much discussion in the literature as to the nature of these ring bodies. Two groups represent the division of opinion on the subject—those who advocate that the ring bodies are remnants of the nuclear membrane, and those who claim that they are artefacts. Cabot's own words in his original paper are as follows: "There is no reason to believe them a mark of cellular degeneration . . . that they have some connection with the nucleus formerly possessed by the cell that contains them seems to me not unlikely . . . their shapes correspond for the most part very well with those of nuclear figures . . . the best hypothesis, therefore, that I can suggest to account for these appearances is that they represent nuclear remains . . . the significance of their red color with Wright's, a stain which never stains any nucleus red, is not clear to me . . . have these figures any practical significance? Not so far as I know."

The following investigators believed the rings to be identical with the nuclear membrane: Schleip,^{2, 3} Pappenheim,⁴⁻⁶ Sluka,⁷ Alder,⁸ Ferrata,^{9, 10} Ferrata and Viglioli,¹¹ Juspa,¹² and Jordan, Kindred, and Beams.¹³ Demel¹⁴ proposed that the Cabot rings are artefacts. Naegeli¹⁵ took a middle ground by stating that Cabot rings are generally considered nuclear membrane remnants, absent in erythroblasts and the blood-forming organs; they are a patho-

¹Cabot, R. C.: *J. M. Res.* 9: 15, 1903.

²Schleip, K.: *Deutsches Arch. f. klin. Med.* 91: 449, 1907.

³Schleip, K., and Alder, A.: *Atlas der Blutkrankheiten*, ed. 3, Berlin, 1936, Urban & Schwarzenberg.

⁴Pappenheim, A.: *Folia haemat.* 9: 318, 1910.

⁵Idem: *Folia haemat. Arch.* 9: 304, 1910.

⁶Idem: *Folia haemat. Supplement*, pp. 48 and 302, 1907.

⁷Sluka, E.: *Deutsches Arch. f. klin. Med.* 93: 577, 1908.

⁸Alder, A.: *Atlas des normalen und pathologischen Knochenmarkes*, Berlin, 1939, Urban & Schwarzenberg.

⁹Ferrata, A.: *Folia haemat.* 9: 268, 1910.

¹⁰Idem: *Le Emopatie* 1: 37, 1933. Soc. Editrice Libreria, ed. 2, Milano.

¹¹Ferrata, A., and Viglioli, G.: *Folia haemat. Arch.* 11: 315, 1911.

¹²Juspa, V.: *Folia haemat.* 17: 429, 1914.

¹³Jordan, H. E., Kindred, J. E., and Beams, H. W.: *Anat. Rec.* 46: 139, 1930.

¹⁴Demel, A. C.: *Haematologica* 2: 125, 1921.

¹⁵Naegeli, O.: *Blutkrankheiten und Blutdiagnostik*, ed. 5, Berlin, 1931, Julius Springer.

logic phenomenon of nuclear dissolution. Schilling¹⁶ believed that it had not been proved that the Cabot-Schleip rings are nuclear remnants. In another publication,¹⁷ he recorded seeing Cabot rings in normal erythroblasts.

Schleicher¹⁸ has rather definitely proved that the Cabot ring bodies are denatured protein configurations and are actually laboratory products. He showed that Cabot ring bodies may be demonstrated by special methods and that they are neither nuclear remnants nor are they identical with the nuclear membrane; they are laboratory creations, the expression of cellular degeneration induced by hemolytic agents; i.e., they are the expression of aggregated and denatured colloid protein. He was able to produce these bodies experimentally. This work was carried out with egg whites on slides. He experimentally produced replicas of Cabot ring bodies in normal mature erythrocytes but not in normal erythroblasts. The creation of the bodies depends upon the physicochemical state of the lipoproteid colloids at the various maturation stages. For fuller information on the method of the experimental investigations, see his original article.

25. Erythrocontes.—Erythrocontes are fine, faintly red-staining rods seen in erythrocytes, especially in the stippled cells of pernicious anemia and other conditions.

26. Metachromatic Substance (Substantia Metachromatica).—Metachromatic substances are fine globules within erythrocytes. They are best demonstrated with supravital stain. They accumulate in one place but may be distributed, often showing lively molecular motility in the dark-field. According to Pappenheim they are lipoid segments. They probably bear some relationship to the archoplasmatic plasmasomes.

27. Schistocytes (Erythrocytic Segments).—Schistocytes are irregular erythrocytic hemoglobin-containing particles, which may be mistaken for blood platelets when unstained. They are never azure red. They represent a degenerative deformity, that is, destruction.

28. Poikilocytes.—Poikilocytes are lobulated, distorted, pear-, oak-leaf-, or thornapple-shaped degenerative forms of erythrocytes. They are found in the blood vessels following severe direct or indirect injury. They represent degenerative malformations, perhaps partially increased vulnerability. They may be artificially produced by careless technic in making the blood film.

29. Endoglobular Degeneration.—Erythrocytes sometimes show irregular unstained areas of different shapes within the cytoplasm. This is probably due to some degenerative influence on the cell, itself, and is referred to as "endoglobular degeneration."

Leukocytes (Schilling Classification)

In order to interpret differential counts of the leukocytes, it is necessary to know certain facts regarding the classification of white blood cells. White blood cells are divided into granulocytes, monocytes, and lymphocytes.

Granulocytes are divided as follows: (1) *Basophilic* leukocytes, or mast cells, are present in the circulating blood in the proportion of 0.5 per cent.

¹⁶Schilling, V.: *Folia haemat.* 14: 95, 1912.

¹⁷Idem: *Das Blutbild und seine klinische Verwertung*, ed. 6, Jena, 1926, Gustav Fischer.

¹⁸Schleicher, E. M.: *J. Lab. & Clin. Med.* 27: 983, 1942.

They are the cells which have an affinity for the basic dyes of the Giemsa and Wright stain. (2) *Eosinophiles* (or *acidophiles*) are present normally in the circulating blood, and comprise 2 to 4 per cent of the leukocytes. They have an affinity for acids, consequently they take the eosin stain. (3) *Neutrophiles* comprise about 60 to 70 per cent of all leukocytes. They require a neutral dye, and consequently stain a pale pinkish lavender or bluish lavender. The reddish purple granules of the basophilic cells are very irregular both in size and arrangement. The eosinophilic granules are large and very regularly distributed throughout the entire cytoplasm of the cells, so that they look very much like small pale orange balls of uniform size placed as close together as possible. The granules of the neutrophilic cells are very fine and scattered unevenly throughout the cells. They stain reddish purple.

According to Schilling's classification, there are four divisions of the neutrophilic cells; namely, myelocytes, juveniles, "stabs" (rod nuclear, staff form, and other terms), and segmented forms. The myelocyte is the youngest form; the segmented form is the most mature. The myelocyte has a round to oval to kidney-shaped nucleus, with large well-filled structures and a distinct trabeculated form. Myelocytes are not found normally in the circulating blood.

The juvenile neutrophilic leukocyte has a sausage-shaped nucleus often with nucleoli¹ usually in the end bulbs. They are identical with Pappenheim's metamyelocytes. They are found only occasionally in the circulating blood in health, in the percentage of 0 to 1. The "stab," or rod nuclear cell, is found normally in health in the percentage of 3 to 5. They have an unbroken, or rod nucleus which is capable of bending or twisting, so that it is seen in many different shapes (see below). The segmented neutrophile is found normally in the percentage of 58 to 66 in the circulating blood. Their nuclei have two, three, or more distinct segments connected by bridges. The juveniles, "stabs," and segmented neutrophiles were formerly referred to as the "polymorphonuclear neutrophilic" cells.

Lymphocytes are present in the percentage of 21-30 in normal blood. They vary in size from a cell smaller than an erythrocyte to one of the diameter of neutrophilic leukocytes. See below for a complete description.

Monocytes are more than twice the size of erythrocytes. They are normally present in the percentage of 4 to 8. It is very important to differentiate these cells from lymphocytes. (See below for complete descriptions of all the cells.)

Description of Leukocytes Stained With Wright-Giemsa Stain

(a) Myelocytic or Bone Marrow Cells

30. **Myeloblasts.**—Myeloblasts are large bone marrow cells with narrow to wide cytoplasm, and a large nucleus. The cytoplasm usually is pale or deep blue with a thready, cloudy design. The nucleus is large, reticulated, and round with one to six definite nucleoli. There are no granules. Frequently the cells show vacuolization. They are oxydase-positive. They are never found in the circulating blood in health. *Micromyeloblasts* are very small

¹It is Schilling's opinion that these are nucleoli. According to many authorities they are not nucleoli, but chromatin granules.

PLATE VIII. ARTEFACTS

1. Design formation of erythrocytes due to the presence of fat on the slide.
2. Partial hemolysis of erythrocytes due to moisture on the slide.
3. Foreign material (fibers) on the slide, stained blue.
4. Crystalline appearance of centers of erythrocytes due to improper fixation of slide, or to the presence of moisture in the cells when cedar oil is added.
5. Precipitate on the center of an erythrocyte.
6. Crenation of erythrocytes due to pressure when making the spread.
7. Carbon particles.
8. Precipitated stain.
9. Blood platelet on an erythrocyte.
10. Erythrocytes stained bright orange, due to acid water used in staining.
11. Erythrocytes stained pale, due to overwashing the slide or using too alkaline a water in staining.
- 12, 13, 14, 15, 16, 17. Effect of acid water on staining of eosinophile, lymphocyte, blood platelets, monocyte, neutrophile, and another lymphocyte.
- 18, 19, 20, 21, 22. Alkaline water staining of neutrophile, two lymphocytes, monocyte, and an eosinophile.
23. Alkaline staining of erythrocytes.
24. Rouleaux formation of erythrocytes, showing the false staining reaction, due either to alkaline water in the staining process, or to improper penetration of the dye into the thick spread.
25. Group of blood platelets, alkaline stained.
26. Neutrophiles and an eosinophile enmeshed in a thick portion of the spread. Erythrocytes partially dissolved, probably due to the alkaline water.
27. Endothelial elements squeezed out of the tissues.
28. Fibrin, due to partially clotted blood.
29. Crushed neutrophiles.
30. Crushed lymphocyte.
31. Crushed eosinophile.
32. Accumulation of leukocytes on the edge of a film, due to improper spreading.



myeloblasts seen only in leukemia. They resemble small lymphocytic cells, but have definite nucleoli, and a very deeply staining blue cytoplasm. The cytoplasm frequently shows projections thought by some authorities to be pseudopodia.

31. Promyelocytes or Premyelocytes.—Promyelocytes are large cells. The cytoplasm is usually pale blue with fine red pro-granulations. The granules may fill the entire cell, or may be scattered around the edge of the cytoplasm. If the cell is in the center of the spread, the granules at times stain blue.* The nucleus is large and round, and usually shows one or more nucleoli. The cell is identified by the fine, red pro-granulation and the definite nucleoli. In leukemia, promyelocytes may be very small cells, closely resembling monocytes. They may be differentiated by the oxydase stain; these promyelocytes are oxydase-positive. They stain very much darker than monocytes, and show nucleoli. The differentiation is frequently very difficult.

32. Neutrophilic Myelocytes.—The cytoplasm of neutrophilic myelocytes varies from very pale blue in the younger stages to pink in the older forms. With Wright stain, it is gray and at times intensely red. The nucleus varies in shape: round, kidney-shaped, or oval. It may or may not contain one or more nucleoli, and is trabeculated. This nuclear structure aids in differentiating it from lymphocytes with azurophilic stippling (*small dots*). In leukemia the granules of the myelocytes are very delicate and difficult to stain. They are purple and pin point in size. They are seen usually only here and there; at times they may be entirely absent. In the blood stream in severe infections, myelocytes usually have a pale blue cytoplasm filled with coarse neutrophilic (purple) toxic granulation. The myelocyte of leukemia has a fairly even periphery, while that of infections is fragile, and consequently may be irregular.

33. Juvenile Neutrophiles or Metamyelocytes.—Juvenile neutrophiles are found normally in the circulating blood in the percentage of 0 to 1 per 100 leukocytes. They are slightly larger than the mature neutrophiles. The cytoplasm varies from a bluish pink to a definite pink or gray, particularly when stained with Wright stain alone. It is much wider than that of the myelocytes. The nucleus is sausage-shaped to almost bean-shaped. It does not stain intensely, and shows little or no chromatin structure, although it is definitely divided into fields. It often contains one or more nucleoli,¹ usually situated in the end bulbs, which are definitely protruding structures. The granules are sometimes definite and sometimes very fine. When they are coarse, they are strikingly responsive to stain; when fine, they stain with difficulty. The same variations in staining and structure may be noted in leukemia and infections as are seen in the myelocytes. They are differentiated from "stab" cells by the pale nucleus without chromatin, the nucleoli (see footnote¹) and the width of the nucleus. "Stab" cells are older, and consequently stain more deeply, have no nucleoli, show dense chromatin structure, and have a fairly narrow nucleus. When a cell has developed beyond the juvenile stage but has not quite become a "stab" (borderline cell), Schilling suggests that it be called a "stab."

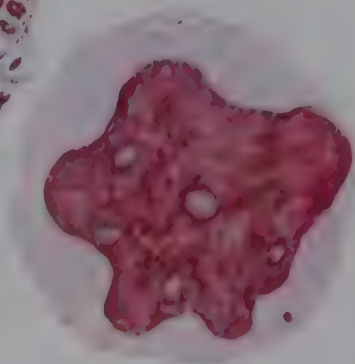
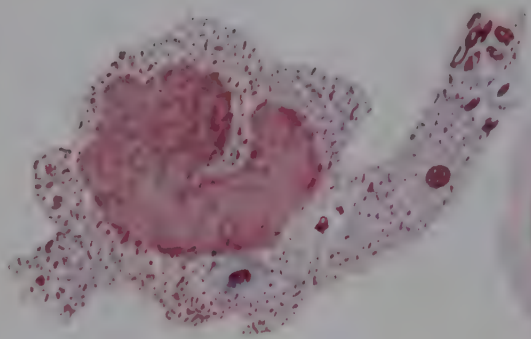
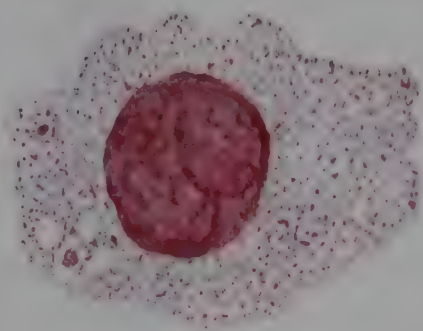
*Observation of Schilling.

¹See footnote on page 637.

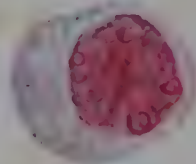
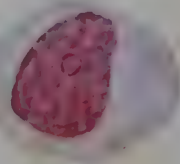
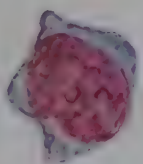
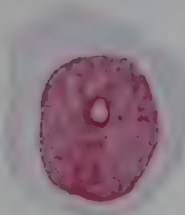
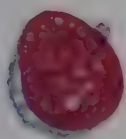
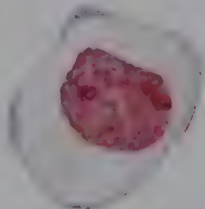
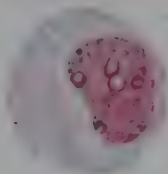
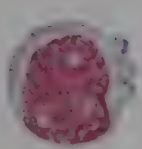
34. **"Stab" or Band or Rod Nuclear Neutrophilic Cells.**—The term "stab," originated by Schilling, is a German word which refers to the ability of the rubberlike rod-shaped nucleus to become bent or twisted. Since there is no handy English equivalent, the German term has been retained. Numerous synonyms are found in the literature, such as "staff cells," "rod nuclei," "nonfilamentous forms," "band cells," etc. "Stab" cells are neutrophilic cells and are found in the normal circulating blood in the percentage of 3 to 5. The cytoplasm is pink or lavender, often gray with Wright stain. The nucleus is a flexible rod, which may be bent in making the film, so that it takes the form of the letters *S*, *T*, *U*, *V*, or *W*. It does not show segmentation, although degeneration forms in which the nucleus is vacuolated or fringed may occur. Any cell which shows even slight segmentation is not considered a "stab" cell. The nucleus contains dark chromatin structure, which must not be mistaken for nucleoli. It stains more intensely than the nucleus of the juvenile cell, and is slightly narrower. It does not contain nucleoli. The granules are usually distinct, very fine, purple-staining stippling, distributed less regularly than the granules of the eosinophiles, but more regularly than those of the basophiles. Under conditions of severe infections, these are very coarse and clumped. (See toxic granules, page 648.) When the cell is very definitely in the borderline stage between a "stab" and a segmented form, that is, if it has developed farther than the "stab" stage, Schilling suggests that it be called a segmented cell. The nucleus of the stab may undergo changes due to mechanical pressure in making the spread. Unusual forms are to be looked for and recognized. It is important to remember, too, that there are *degenerative* forms of the stab cells. The degenerative forms show small band forms of nuclei, irregular, hyperchromatic, pyknotic, and structureless. They are easily broken up in making the preparation. There is a tendency toward vacuolization and diminished resistance. They are a result of a toxic element or infection.

35. **Segmented Neutrophiles.**—Segmented neutrophiles are found normally in the circulating blood in the proportion of 58 to 66 per 100 leukocytes. The diameter of the cell is about one and one-half that of an erythrocyte. The cytoplasm is usually a clear pale pink or lavender. The nucleus is usually divided into two to five irregular segments which are connected in a chain by fine threads or bridges. They show deep-staining chromatin structure, and no nucleoli. The granules are fine, light-refracting, pale reddish violet, except in severe infections, when they are coarse and massed in irregular clumps. In certain conditions (neutropenia with right shift), the neutrophiles may be very large, ovoid, coarsely granulated, and hypersegmented; that is, have six or more lobes to the nucleus. The presence of such cells must always be noted on the report of the differential count.

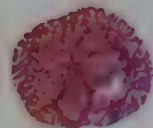
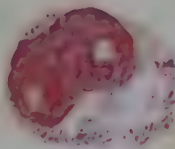
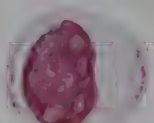
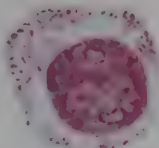
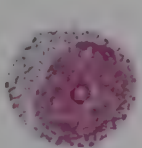
36. **Eosinophiles.**—Eosinophiles are found normally in the circulating blood in the proportion of 2 to 4 per 100 leukocytes. The clear white or pale blue cytoplasm is composed of many highly light-refracting granules which stain a faint orange to a decided orange with the Giemsa stain. With the Wright stain, they are more intensely stained. They are usually situated so close together that they give the appearance to a casual observer of an



MEGAKARYOCYTES

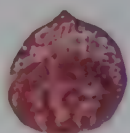
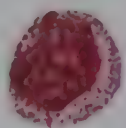
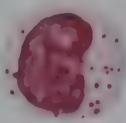
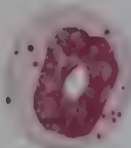
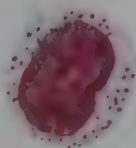


MYELOBLASTS

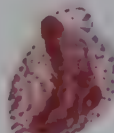
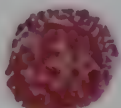
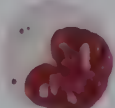
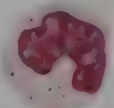
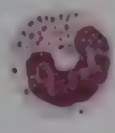


PROMYELOCYTES

NEUTROPHILES



MYELOCYTES



JUVENILES

A. G. ERSKINE

almost uniformly orange stained cytoplasm. The granules may be rendered more pronounced by partially closing the iris diaphragm. In young eosinophilic cells, or in mature cells if alkaline water is used in staining, there are frequently found deep blue granules. Sometimes the entire cytoplasm stains a uniform gray color, but the identification of the cell can still be made by the regularity of the granules and the shape of the nuclei. Bright crystal-like granules may also be seen. This is an artificial picture due to the highly light-refracting characteristic of the granules.

Eosinophiles are differentiated as eosinophilic myelocytes (if they have round or oval nuclei), eosinophilic juveniles (if the nuclei are as described in 33), eosinophilic "stabs" (with the typical "stab" nucleus), or segmented eosinophiles. It is customary not to classify the eosinophiles except as myelocytes and segmented eosinophiles (simply eosinophiles).

The nucleus of the mature eosinophile usually has two segments, double pear-shaped. There are seldom more than three or four segments, although it is possible to find them. Eosinophiles retain their characteristics in the thick drop. To intensify the orange color of the granules, make the staining water slightly acid.

37. Basophiles or "Mast" Cells.—Basophiles may also be divided into basophilic myelocytes and mature cells. The mature forms are found normally in the circulating blood in the proportion of 0 to 1 per 100 leukocytes. The cytoplasm is usually very narrow, and stains deep reddish purple with the Giemsa stain. With the Wright stain the cytoplasm is often blue. It is composed almost entirely of irregular basophilic fragments, which usually stain intensely. These fragments are very highly soluble in water, and therefore drop out of the cytoplasm during the staining process, leaving the cell appearing highly vacuolated. The coarse red-purple granules are dispersed irregularly throughout the remaining cytoplasm. If the granules do not dissolve, they stain intensely reddish purple, and are unevenly distributed throughout the cytoplasm. With Wright stain the granules stain a dark blue-purple. The nucleus is bizarre, lobulated, and occupies most of the cell. It stains a deep reddish purple with the Giemsa stain and an intense bluish purple with the Wright stain.

(b) Cells From the Reticuloendothelial System

38. Monocytes.—Monocytes were formerly called large mononuclear cells and transitionals. They are found normally in the circulating blood in the proportion of 4 to 8 per 100 leukocytes. They are usually much larger than the granulocytes, but may be as small as a small lymphocyte. The cytoplasm is usually very wide, is smoky blue to pale pinkish violet or blue, and is frequently covered with a very fine pink dust. It may or may not show vacuoles. The cell shows a very irregular outline, which helps differentiate it from lymphocytes. With Wright stain the differentiation from lymphocytes is very difficult, and at times impossible. The nucleus is fairly large, and may vary from oval to bean-shaped, or kidney shape, or may even show lobulation. Occasionally it closely resembles the nucleus of the juvenile neutrophile. It can easily be differentiated by the color and structure of the cytoplasm.

Sometimes a sausage-shaped nucleus is seen, and at times nucleoli are distinctly visible. The edges are seldom, if ever, smooth; the contour appears wrinkled instead of smooth or flat. Monocytes are usually granulated, the cytoplasm being filled with very fine pink dustlike granules, which, when stained intensely, may be mistaken by the beginner for neutrophilic granules. They are, however, less distinct than neutrophilic granules, and are possible of differentiation. Very large phagocytic monocytes have been called *macrophages*.

Unlike the lymphocytes, the monocytes do not show a perinuclear unstained zone. Differentiation of these cells from lymphocytes is made as follows:

1. Monocytes are usually larger than lymphocytes, but the size is not a point in differentiation if all other characteristics show the cell to be distinctly monocytic.

2. The nucleus of the monocyte is wrinkled; that of the lymphocyte is smooth and solid, and usually shows deeply stained chromatin material. The monocyte occasionally shows nucleoli.

3. The structure of the monocyte is reticulated and more delicate than the structure of the lymphocyte; therefore, the cell stains paler than the lymphocyte.

4. The monocyte tends to show great variation in the shape of the nucleus.

5. The lymphocyte usually shows a clear, unstained perinuclear zone, whereas the monocyte is usually devoid of such a zone.

6. The monocyte shows a pink, powdery granulation, while the lymphocyte is either without granules, or shows azurophilic stippling.

7. The cytoplasm of monocytes is smoky blue, gray, or lavender, while that of lymphocytes is a definite clear pale or deep blue.

39. **Endothelial Cells.**—Endothelial cells are long, caudate cells, frequently have a conspicuous oval, excentric, finely designed nucleus. At times nucleoli are visible. The cytoplasm is very delicate, and if visible, stains either very pale pink or very pale blue. There seem to be fine pink granules or dust throughout the irregular cytoplasm, although this may be absent. They may originate from a skin wound, but if they appear in large numbers they probably represent emigrated reticuloendothelia, or according to Hess, vascular endothelia of local origin. They are relatively frequent in endocarditis ulcerosa and severe blood infections. They are closely related to the monocytes.

40. **Monoblasts.**—Monoblasts are immature monocytic cells and are larger than mature monocytes. They are never found normally in the circulating blood. They are irregular in shape. The nucleus is in the form of an irregular crescent or is broad and elongated. It might be bent over itself several times, giving the impression of lobulation. It has a loosely woven chromatin network, and often stains paler than lymphoblasts and myeloblasts. The cytoplasm is more abundant and less basophilic than that of lymphoblasts. Azure granules may be seen; vacuolization is common. They exhibit lively ameboid movements and may be either oxydase-positive or negative.

NEUTROPHILES

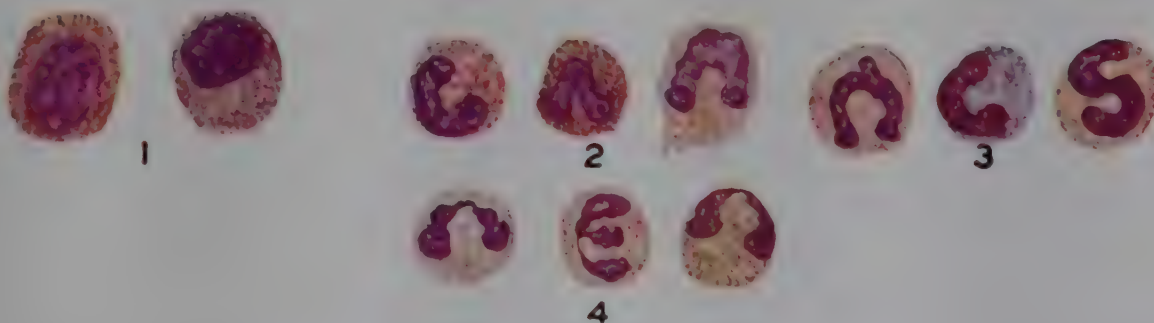


"STABS"



SEGMENTS

EOSINOPHILES



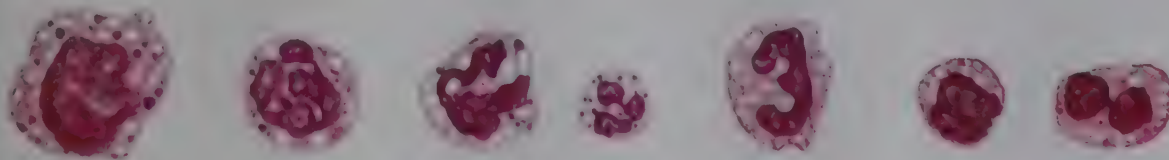
1. MYELOCYTES

2. JUVENILES

3. "STABS"

4. SEGMENTS

BASOPHILES



A. G. GOSKIN

A.

PLATE X.—LEUKOCYTES. GIEMSA STAIN. $\times 950$.

A.—Basophilic myelocyte.

(c) Lymphocytic Cells

41. **Lymphoblasts.**—Lymphoblasts are never found normally in the circulating blood. The cytoplasm is usually deep blue with a threadlike cloudy design appearance. It is usually narrow and has a smooth edge. At times, however, cytoplasmic projections, said to be pseudopodia, are observed. The nucleus is large, reticulated, and may have one or two nucleoli, occasionally three. There are no granules. The cells are oxydase negative.

42. **Lymphocytes.**—Lymphocytes are found normally in the circulating blood in the proportion of 21 to 30 per 100 leukocytes. Some authorities consider 35 per cent lymphocytes a normal finding. They have been grouped as large and small lymphocytes, the large lymphocytes comprising about 6 per cent of all the blood leukocytes. The cytoplasm varies from very pale, almost invisible, clear light blue to deep blue, always clear. The width of the cytoplasm varies from a very narrow band to a very broad, smooth cytoplasm. Occasionally no cytoplasm is visible. There usually is an unstained perinuclear zone clearly visible. The nucleus is always stained deep blue or violet. It may show deep-staining chromatin structure, although the general appearance is smooth. It may be round, oval, or indented. It may be central or excentric. Sometimes the nucleus shows distinct lobulation or segments (Rieder forms). These forms are pathologic. There are no granules, although azurophilic stippling is not uncommon. For differentiation from monocytes, see preceding description.

Age of Lymphocytes.—Some very interesting facts concerning the lymphocytes of the peripheral blood have been discussed by Wiseman.¹ The age of the lymphocytes has been utilized by Wiseman and others as a criterion of progress or recession of tuberculosis in man in the differential blood picture. The following characteristics of blood cells are useful in estimating the age of lymphocytes.

1. *Basophilia.*—In 1898 Pappenheim² noted that the cytoplasm of young myelocytes is very basophilic. We know that the "reticulation" in young red blood cells is merely a precipitation of basophilic substance; and in the reticulated cell counts we have an example of the use of the presence of basophilia as an index of immaturity.

2. *Mitochondria.*—That mitochondria are present in large numbers during the maturation of young blood cells other than lymphocytes is generally accepted. In an excellent review, Cowdry³ states that senescence is associated with diminishing number of mitochondria in the cytoplasm of cells in general. Increased numbers of mitochondria are associated with the activity of secretory cells.

3. *Size of the Cell.*—The size of the cell has been used by hematologists as a criterion of the age of lymphocytes. This is uncertain, however, as proved by the fact that some have regarded the large and some the small lymphocyte as the younger cell, while others hold that size is no criterion of age at

¹Wiseman, Bruce K.: *J. Exper. Med.* 54: 271, 1931.

²Pappenheim, A.: *Virchows Arch. path. Anat.* 151: 89, 1898.

³Cowdry, E. V.: *Carnegie Inst. of Wash., Pub. No. 25, Contributions to Embryology* 16: 229, 1925.

all. It is significant that most of the investigators studying fixed, stained specimens adhere to the belief that the large cells are the older types, whereas those who follow the supravital technic hold the reverse.

4. *Miscellaneous Features Characterizing Lymphocytes.*—Here may be listed motility, vacuoles, chromatin content of nucleus, proportion of nucleus to cytoplasm, shape of nucleus, and azurophilic granulation.

Wiseman, in attempting to evaluate the age of lymphocytes, studied normal adult human and rabbit bloods. As a result of his research, he states that basophilia is manifestly a criterion of youth, but the absence of it in a given cell does not necessarily mean that the cell is mature. No single criterion enables one to pronounce upon this point. In order to determine the age, all the factors must be taken into account, but basophilia is the most constant and reliable criterion. Size is not strictly an attribute of age in lymphocytes. There is no correspondence in the size of lymphocytes in supravital films and in fixed specimens obtained by the "cover glass" method. There is a change of size during fixation. Although lymphocytes of intermediate and large size may be of any age, in supravital preparations the majority are young cells, whereas the chromatin content of the nucleus does appear to be greatest in any age in specimens examined by either technic. The shape of the nucleus of the lymphocyte, the presence of indentation and its degree, does not bear any relationship to the number of mitochondria or the degree of basophilia, and hence is unrelated to age, in direct contradiction to Arneth's hypothesis¹; whereas the chromatin content of the nucleus does appear to be greatest in those cells deficient in the cytoplasmic criteria of youth. In general, in the large lymphocytes, the greater amount of cell occupied by the nucleus, the more basophilic the cytoplasm. Nonstaining, refractive vacuoles are found only in cells devoid of both mitochondria and basophilic substance; whereas the presence and number of neutral red vacuoles do not seem to bear any relationship to either of these features.

For a description of lymphocytes in infectious mononucleosis, see page 821.

43. **Plasma Cells.**—Plasma cells are rarely found in the circulating blood in health. The cytoplasm stains intensely blue with a fine reticular blue structure often showing vacuoles. The nucleus is usually excentric, and is surrounded by an unstained clear perinuclear zone. Because of the dark spots of the nucleus, these cells are often confused with megaloblasts. Although the nucleus somewhat resembles that of a megaloblast, the cell can be differentiated by its perinuclear zone and the structure of the cytoplasm. There are no granules.

Origin of the Plasma Cell.—

The origin of the plasma cell has not been definitely determined, although there has been a great deal of investigation and speculation on this subject. Michels² has discussed the various theories of origin, morphogenesis, function, and development of the plasma cell. He summarized the theories of origin as follows: (1) A histogenous origin from connective tissue cells, including

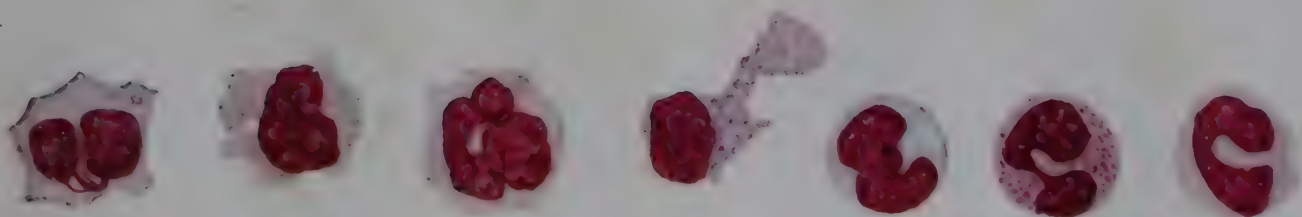
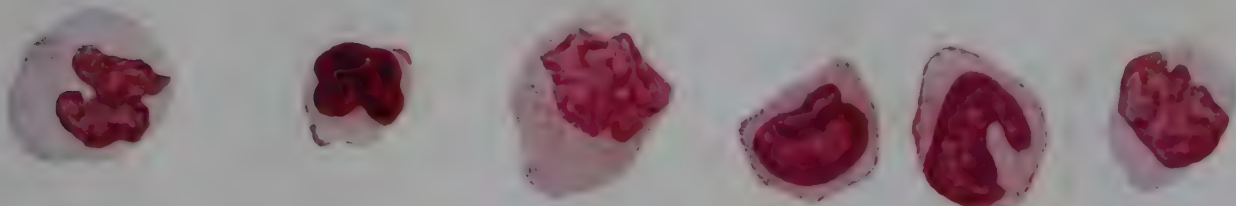
¹Arneth, J.: *Die qualitative Blutlehre*, Leipzig, 1920, W. Klinkhardt.

²Michels, N. A.: *Arch. Path.* 11: 775, 1931.

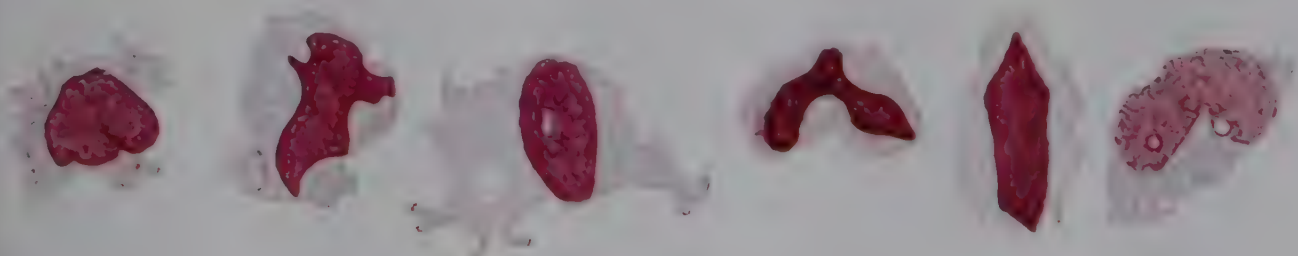
LEUKOCYTES

GIEMSA STAIN

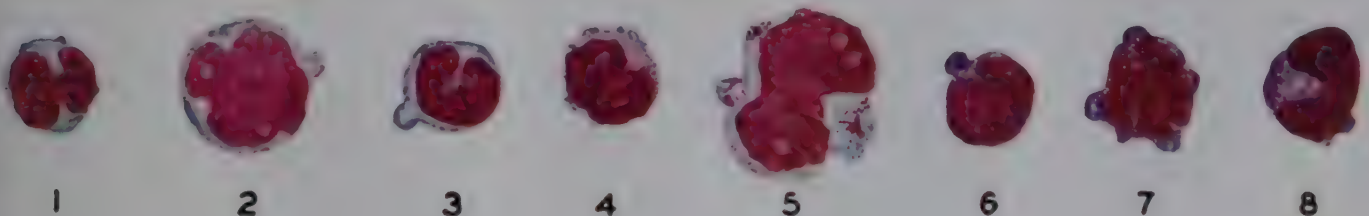
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MONOCYTES



ENDOTHELIOID MONOCYTES



1 - 3 MICROMYELOBLASTS

4 - 8 ATYPICAL PROMYELOCYTES

A. G. ERSKINE

tissue lymphocytes, fibroblasts, clasmatoocytes, resting wandering cells, adventitial cells, and hemohistioblasts. (2) A hematogenic origin from emigrated lymphocytes. (3) Mixed origin from emigrated lymphocytes (monocytes) or pre-existent tissue lymphocytes. (4) An origin from immature blood cells (myeloblasts, hemoblasts, erythroblasts, granuloblasts) through aberration or abortion. Michels favors the third theory. The observations of Lowenhaupt³ are in favor of a reticuloendothelial origin of plasma cells. She found plasma cells arising from sinus endothelial cells of seven spleens and concluded "An origin from histiocytic type cells is indicated by the sites of proliferation in the spleen, liver, and lymph nodes, by the type of hepatic infiltration, and is suggested by the invasion of bone." In the case described by Moss and Ackerman, sections taken from the spleen, liver, and lymph nodes tended to support Lowenhaupt's findings.

44. Auer Bodies.—These bodies were described by Auer⁴ in the large lymphocytes of a case of acute lymphocytic leukemia. They appeared as highly refractive rods, staining red with Giemsa or Wright stain. These rods are from 1 to 5 microns long and 0.75 micron thick. The same observation has been made by Pappenheim⁵ and others. Naegeli claims that they occur only in acute myelocytic leukemia.

It has been claimed by many writers that these Auer bodies are similar to the Kurloff bodies in guinea pigs. Rosenthal believes that they are diagnostic of acute myelocytic leukemia, although Lonero and Quarenta⁶ reported their presence in chronic myelocytic leukemia. Levine⁷ and Forkner⁸ found them in monocytic leukemia. We have found them in myelocytic, lymphocytic, and monocytic leukemia.

(d) Megakaryocytes and Blood Platelets

Megakaryocytes were formerly considered the parent cells of the granulocytes. They are giant bone marrow cells never seen in the blood stream in health, and very rarely found in the blood stream in disease. They are seen pathologically in the spleen and in the lungs. They are very large, finely granular, faintly colored cells. The cytoplasm is pale blue or lavender. The nuclei show bizarre, antlerlike deep lobulations; they contain one, two, or more, sometimes as many as twelve, of these nuclei. They are covered with a faint azurophilic dust.

Megakaryocytes are rarely found in the circulating blood. They have been seen in leuko-erythroblastosis, myelocytic leukemia,⁹ erythremia, Hodgkin's disease, and in cases in which leukocytosis was present due to infection. They have been seen in acute leukemia, aleukemia leukemia, purpura hemorrhagica, pernicious anemia, and plumbism. In megakaryocytic leukemia, megakaryocytes have been noted in the blood. Megakaryocytes have been found packed in the lung capillaries in conditions due to abnormal or immature bone marrow physiologic processes.¹⁰

³Lowenhaupt, E.: *Am. J. Path.* **21**: 171, 1945.

⁴Auer, J.: *Am. J. Med. Sc.* **131**: 1002, 1906.

⁵Pappenheim, A.: *Berl. klin. Wchnschr.* **45**: 60, 1908.

⁶Lonero, G., and Quarenta, L.: *Rinascenza Med.* **6**: 7, 1929.

⁷Levine, V.: *Folia haemat.* **52**: 305, 1934.

⁸Forkner, C. E.: *Arch. Int. Med.* **53**: 1, 1934.

⁹Downey, H., Palmer, M., and Powell, L.: *Folia haemat.* **41**: 55, 1930.

¹⁰Minot, G. R.: *J. Exper. Med.* **36**: 1, 1921.

Blood platelets are described on page 554.

45. **Megakaryoblast.**—These are cells about twice the size of myeloblasts with a blue nongranular cytoplasm and a large somewhat irregular single nucleus, which at times is kidney shaped, with numerous nucleoli. These cells do not produce granular or nongranular platelets either normally or in cases of idiopathic thrombocytopenic purpura. In normal preparations they comprise less than 1 per cent of all the megakaryocytes.

46. **Promegakaryocyte.**—These cells are usually about the same size as the megakaryoblast, although occasionally much larger, with scant dark blue cytoplasm and a dense nonlobulated or partly lobulated nucleus with heavy chromatin. These forms frequently have plateletlike bodies at the periphery of their cytoplasm and rarely nongranular cytoplasmic processes. When both these details are present, the cell is designated as one with granular platelet production. Cells with nongranular processes are absent or rare in normal cases but are frequent in cases of purpura. Normally, promegakaryocytes make up about one-third of the total number of megakaryocytes.

47. **Lymphoid Megakaryocyte.**—This is a large cell with basophilic cytoplasm, usually without granules, and a nucleus which is relatively small in comparison with the entire cell and usually distinctly lobulated.

48. **Intermediate Forms.**—These cells are intermediate in size or in maturation between the relatively small promegakaryocyte and the huge adult forms. The cytoplasm is very heavily granulated. Platelet formation may or may not be present.

49. **Adult Megakaryocyte.**—This cell is of variable size, but usually very large, containing a single large multilobed nucleus with increasing density as it matures. The cytoplasm varies in color from blue to pink and contains a variable number of characteristic azurophilic granules grouped at first in the perinuclear zone. Typical platelets which are almost always granular are frequently found in pseudopodlike structures grouped in masses about the periphery of the cell. This cell is probably formed by nuclear fusion from the polykaryocyte.

50. **Prepolykaryocyte.**—This is a mononuclear cell which tends to occur in clusters. There is an abundant vacuolated blue cytoplasm without granules and a clear reticulated excentric round nucleus with one or more nucleoli. The cells are seen normally and do not appear to be increased in purpura.

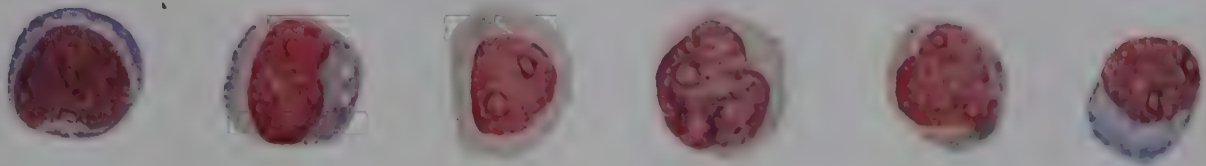
51. **Polykaryocytes.**—These are probably fused syncytia of the above; that is, a large number of individual nuclei lie within one cytoplasmic body. These cells are probably identical with the osteoclasts.

52. **Degenerated Forms.**—In these cells the cytoplasm is either homogeneous and hyaline in appearance, or there is marked vacuolization of the cytoplasm, or the nucleus is hyperlobulated and its cytoplasm nongranular.

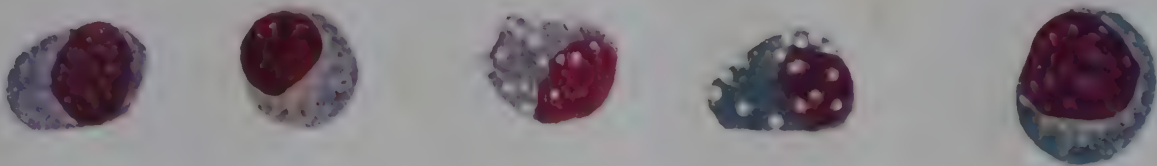
It must be recognized that the relationships of the various types of cells to each other may be more artificial than real, since they are based on a study of what appear to be transition forms. Because of this uncertainty, the megakaryoblasts and promegakaryocytes may be designated as “young forms,” and the lymphoid megakaryocytes, intermediate forms, and adult types as “adult forms,” with a separate designation for the degenerated cells.

LEUKOCYTES GIEMSA STAIN

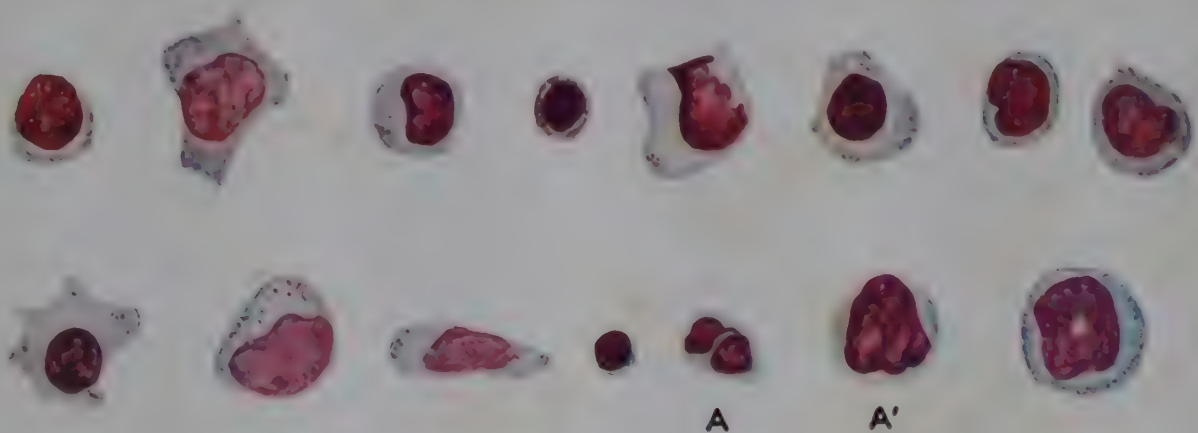
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LYMPHOBLASTS



PLASMA CELLS



LYMPHOCYTES, INCLUDING RIEDER CELLS (A, A')

A.G. Erskine

(e) Miscellaneous Structures in Blood Cells

53. **Nuclear Fragments.**—Fragmentary nuclear structures have been called by several terms. They are disintegrated nuclear masses, and closely resemble endothelial cells. The edges of the nuclei of these fragments, however, are not definite, but are frayed, while nuclei of endothelial cells have a very definite edge. They may be produced by undue pressure on the cover glass while making a blood spread, or they may originate pathologically. They are seen in great numbers in lymphocytic leukemia.

54. **Sediment of Stain.**—Sediment of stain can be recognized easily because of its lack of structure. It is often seen when films are made too thin and then stained with Wright stain, or when the water is added too slowly in staining with Wright stain. Some stains precipitate upon standing, and the precipitate appears on the blood spread. This is especially to be avoided when making blood platelet and reticulocyte preparations.

55. **Portions of Cytoplasm of Cells.**—At times cytoplasm of cells will be seen without the nuclei, especially granules of neutrophiles and eosinophiles. These must be studied and identified as such.

56. **Cells With Two or More Nuclei.**—Schilling called attention to a binucleated myeloblast or lymphoblast which is seen in cases of leukemia. These are cells with two nuclei without any signs of mitosis. They are giant cells, with grayish, thready, smooth cytoplasm. The nuclei have the same structure as those of myeloblasts. At times plasma cells are seen with multiple nuclei.

Qualitative Changes in the Leukocytes

The Arneth Classification of Normal Leukocytes

In addition to the regenerative changes in the nuclei of the leukocytes, described by Arneth and later by Schilling, many hematologists have called attention to qualitative changes in the cytoplasm of these cells, under the influence of various offending factors. The Arneth classification of normal leukocytes described by him in 1904 is as follows:

CLASS 1			CLASS 2			CLASS 3				CLASS 4				CLASS 5					
M	W	T	2K	2SS	1K1S	3K	3S	2K1S	2S1K	4K	4S	3K1S	3S1K	2K2S	5K5S	4K1S	3S1K	3S2K	ETC.
-	-	4	-	17	4	2	13	14	19	4	-	7	4	8	2	1	1		

Class 1 was subdivided into three groups (not in normal blood):

- 1. M-cells Myelocytes, single round nuclei.
- 2. W-cells Neutrophiles with slightly indented plump nuclei.
- 3. T-cells Neutrophiles with deeply indented nuclei (4-9% neutrophiles).

Class 2 was subdivided into three groups:

- 1. 2 K-cells Neutrophiles, with two rounded parts.
- 2. 2 S-cells Neutrophiles, with two elongated parts.
- 3. 1 K 1 S-cells Neutrophiles, with a single round nuclear part and an elongated portion.

Class 3 was divided into four sections (33-48% normal blood):

- 1. 3 K-cells Three round nuclear parts.
- 2. 3 S-cells Three nuclear strands.
- 3. 2 K 1 S-cells Two round nuclear parts, one strand.
- 4. 2 S 1 K-cells Two nuclear strands, one round portion.

Class 4, five kinds (9-23% normal blood):

1. 4 K.
2. 4 S.
3. 3 K 1 S.
4. 3 S 1 K.
5. 2 K 2 S. (2-4% normal blood.)

Class 5, various combinations of nuclear round and stranded portions.

He described a left shift of these cells under the influence of infection. Schilling, as already noted, modified and simplified this cumbersome classification, giving a neutrophilic classification of myelocytes, juveniles, stabs, and segmented cells.

The Barta Classification of Granules and Cytoplasm

Various Italian workers have repeatedly called attention to changes in the cytoplasm of cells, as well as changes in nuclear forms. Cesaris-Demel¹ attempted to draw clinical conclusions from these changes. He utilized methods of supravital staining to demonstrate these changes. He assumed that these changes were *degenerative*. Hirschfeld² was the first German authority to speak of this finding. He described irregularly enlarged, basophilic granules. Schilling noted the same findings, using the dark-field method, thus avoiding any possibility of changes due to the dyestuffs used in blood staining. He called these changes "toxic degenerative." Naegeli and his followers have gone still farther in describing and explaining these changes. Gloor³ has published a very complete study of the granules, as seen in the vast hematologic service of Naegeli at Zurich. Barta⁴ reported a still more complete study of the nature of these granules.

Granules	Cytoplasm
I. Regenerative Progranules	I. Vacuoles
II. Normal Granules	(a) autolytic
III. Pathologic Granules:	(b) digestion, a secondary change to phagocytosis
1. Albuminous granules	II. Basophilia
(a) endogenous (necrobiosis)	(a) immature
(b) exogenous (identical with Freyfeld and Mommsen granules)	(b) alteration of cells
2. Fatty granules	(a) primary
(a) true fat (endogenous)	(b) secondary
(b) excess stored or extraneous	III. Doehle Inclusion Bodies
3. Glycogenic granules	Basophilic punctation of protoplasm,
(a) degeneration (endogenous)	NOT identical with pathologic granules.
(b) degeneration (exogenous)	

Using neutral red as a supravital stain, normal granules are yellow-brown; pathologic granules are intensive orange-red.

Naegeli and his school regard all these changes as pathologic granules, but Barta believes that they represent a change due to exaggerated function of the leukocytes. He believes that they are somehow concerned with the destruction of bacteria, following upon the old phagocytic theory of Metchnikoff.

¹Cesaris-Demel: Gior. d. r. Accad. di med. di Torino, 1906, 1907.

²Hirschfeld: Berl. klin. Wchnschr. 1901.

³Gloor, W.: Monograph, Leipzig, 1929, Geo. Thieme.

⁴Barta, I.: Folia haemat. 41; Nos. 1 and 2, 1930.

Schilling contends that the change is *colloidal*, due to changed permeability, or a functional reaction; Naegeli and Gloor insist upon a "toxic" effect as the true explanation.

Barta introduced a new summary of leukocytic changes, utilizing the Schilling claims as well as retaining his own:

• The Barta Classification of Pathologic Changes in Leukocytes

I. Regenerative Changes:

(a) regenerative nuclear shift.

(b) protoplasmic immaturity, basophilia, azurophilic progranules.

II. Degenerative Changes:

(a) degenerative nuclear shift (karyolysis, pyknosis, degenerative shift).

(b) degenerative protoplasmic changes (plasmolysis, necrobiosis, autolytic enlargement and vacuolization, endogenous products, etc.).

III. Functional Changes:

1. Phagocytic changes (parasites, etc.).

2. Accumulation granules:

Albumin granules, identical with Freyfeld and Mommsen.

Fat, glycogen, dye granules, etc.

Regardless of the controversy as to just what the nature of toxic or pathologic granules of leukocytes really is, how important are they in diagnosis and prognosis? They are particularly important in the diagnosis and prognosis of staphylococcal and streptococcal sepsis. Gloor maintains that their presence in blood spreads in staphylococcal infections indicates a flooding of the system, or a metastatic process. He reported a great difference in the number of toxic granules in widespread streptococcal sepsis and endocarditis lenta, very few in the latter, many in the former. He claims that the greatest number of toxic granules are seen in croupous pneumonia. Barta's conclusions are that the granules indicate a humoral or phagocytic reaction, that they do not parallel the clinical symptoms, but are subordinate. Estimation of these granules does not constitute a substitute for the blood picture estimation, but serves to round it out. With the clinical symptoms and blood picture, they may be used to complete the diagnostic and prognostic summation.

Inclusions in Leukocytes

Doehle's Inclusion Bodies.—Doehle's inclusion bodies of scarlet fever, with Giemsa stain, appear as pale blue, small irregular clumps in the cytoplasm of neutrophils. They stain reddish yellow with pyronin. They occur sometimes singly, sometimes in large numbers, and vary in size up to about 2 microns. Occasionally they have a thready structure. Matis thinks they are enlargements of Freyfeld's toxic granules, while Freyfeld, himself, differentiates them. They are found very frequently in scarlet fever, so that their absence is a nonspecific general indication against the diagnosis. They are also seen in many highly regenerative-degenerative neutrophilic conditions, especially infection. They belong to the intergranular substance, perhaps to the spongioplasm.

The fat globule inclusions of Cesaris-Demel appear as light vacuoles in Giemsa preparations.

Intergranular vacuoles are found frequently in severe toxic infection, in acute yellow atrophy of the liver, in purpura, and in endocarditis lenta.

Method of Demonstration of Toxic Granules

Although a combined Wright and Giemsa stain has been recommended by Gloor, Schilling, and others, Barta prefers fixation with methyl alcohol, followed by acid or basic stains, also separate stains with supravital methods.

For acid staining, use 1% eosin, erythrosin, acid violet, or picric acid. This stain does not give a sharp enough picture.

Basic stains are better: Loeffler methylene blue.

Stain 2 minutes. Pour off and dry.

The structure of the nucleus is easily recognizable; chromatin is intensively blue, parachromatin paler. Protoplasm is pale with bluish spongioplasmatic reticulum. The normal granules are unstained, while abnormal granules are blue-violet to dark blue.

Supravital Method.—Using the brilliant cresyl blue stain, the normal granules show a blue-violet tone; between them we see variable sized metachromatic small and misshapen granules.

Making and Staining of Thick Drop Preparations

The thick drop method of examination of blood is a very important procedure in searching for parasites, polychromasia* of the red cells, basophilic punctation, and counting eosinophiles.

Parasites of malaria, filaria, trypanosomes, etc., may be found more readily in the thick drop than in the blood film. Often these parasites may be seen in the thick drop, but not in the spread. The reason for this is that every field in the thick drop preparation is equivalent to 50 fields in the spread. Thus examining 4 fields in the thick drop is equal to examining 200 fields in the spread. The erythrocytes are dissolved by the water in the diluted Giemsa stain, making it easy to see blood parasites and structures of young erythrocytes. The eosinophiles are very definite, the granules being bunched together. Basophiles appear as a group of fibrin needles around a circular solidly stained center, the whole structure stained purple-red. Neutrophiles are typical, except that the cytoplasm has dissolved. The nuclei and granules, however, remain. In other words, all substances in the blood that are soluble in water are dissolved in the thick drop staining process. The other structures remain as they are.

Technic.—

Place 2 drops of blood on a glass slide.

Spread the 2 drops in a circle to 3 times the original diameter of the drop.

Allow to dry thoroughly either in an incubator (after preliminary drying in the room) for ½ to 3 hours, or at room temperature. Do not leave it in bright sunlight, or anywhere where dust may settle on the slide. *Do not fix.*

Place the slide on a staining bridge. Gently cover with Giemsa stain, taking care not to loosen the drops from the slide.

*In connection with the thick drop, the term "polychromasia" is loosely used. It refers to the reticula of immature erythrocytes left on the slide during the staining process. Such cells would be classified as polychromatic erythrocytes in the fixed film; hence, the term "polychromasia."

Allow the diluted stain to remain in contact with the drops of blood for 3 minutes, or until the hemoglobin begins to dissolve. This can be detected by holding a piece of white paper under the slide. The hemoglobin will be seen flowing through the diluted stain. Then gently tilt the slide, and allow the stain to drain off. Re-cover with diluted Giemsa stain, taking care not to dislodge the drops of blood.

Stain for 30 minutes.

Remove the stain by gently tilting the slide.

Wash very carefully with neutral distilled water, so as not to remove the blood drops from the slide, and allow to dry by standing the slide on end. *Do not blot.*

Examine with the oil immersion objective.

Modified Method of Staining Thick Drops for Examination for Malarial Parasites¹

Mix Giemsa stain with buffer solution pH 7.0 to 7.2 or neutral distilled water in the proportion of 1 drop of Giemsa stain for each 2 drops of buffer. Mix in a test tube or small cylinder.

Place just enough stain on the thick drop to cover it and allow to remain for 3 minutes.

Wash either by immersing in a jar of neutral distilled water, or in the usual manner of washing a slide.

Combined Thick Drop-Thin Film Method (Agress Modification)

Place a drop of blood on one end of a glass slide and spread to about 2 or 3 times its diameter in a circular manner, using the puncture point or the corner of another glass slide.

Make a thin spread of blood on the rest of the slide in the standard manner.

Separate the area of the thick drop and the thin spread by drawing 2 or 3 lines between them with a wax pencil.

Either place the slide in a 37° C. incubator for ½ hour, or allow to remain at room temperature for 1 hour.

Add Wright stain for 2 minutes to the thin spread *only*.

Dilute with neutral distilled water or buffer for 2 minutes, taking care that this stain does not run over onto the thick drop.

Pour off.

Flood the entire slide, including the thick drop, with diluted Giemsa stain (freshly prepared, using 1 drop of stock Giemsa stain to 3 c.c. of neutral distilled water or buffer solution).

Allow to remain on the slide for 30 minutes.

Wash and air dry.

Rapid Thick Drop Staining Method (Field)

A rapid method of staining thick drop preparations has been developed by Field.² The fundamental theory of this method was reported by Simons in 1938 and by Pampani in 1938, namely, that the hemoglobin is used to provide a color contrast, or in effect, to act as a counterstain. The stained parasites and the leukocytes are contrasted against a background of laked red cells. This work was confirmed by Roberts in East Africa. The original method of Simons has been adapted by Field to the Romanowsky principle and stains the chromatin differentially in two seconds. The method embodies rapid staining with isotonic aqueous solutions of stain.

¹This is the method used at the Ryder Memorial Hospital, Humacao, Puerto Rico. A. W. Vigo: Lab. Digest 5: 2, 1941.

²Field, J. W.: Tr. Roy. Soc. Trop. Med. Hyg. 35: 35, 1941.

Technic of Making the Thick Film.—The thick film is made about the size of a shilling or a quarter dollar. It is about ten to fifteen times the thickness of an ordinary thin film. Make it thick enough so as barely to read the dial of a watch through the film.

Stain as soon as the film is dry. This drying may be accelerated by a hot air current from an ordinary hair dryer. No fixation is used. Freshly prepared films stain better than those several days old.

Stains.—

Solution A (used for 1 second).—

- 0.8 gm. methylene blue
- 0.5 gm. azure I*
- 5.0 gm. disodium hydrogen phosphate (Na_2HPO_4 , anhydrous)
- 6.25 gm. potassium dihydrogen phosphate (KH_2PO_4 , anhydrous).
- 500.0 c.c. distilled water.

Dissolve the phosphate salts first. Effect solution of the dyes by grinding in mortar, using a small amount of the phosphate solvent at a time. Allow to stand for 24 hours, then filter. Stain is then ready for use.

Solution B (1 to 3 seconds)

- 1.0 gm. eosin (yellowish, water soluble)
- 5.0 gm. disodium hydrogen phosphate (Na_2HPO_4 , anhydrous)
- 6.25 gm. potassium dihydrogen phosphate (KH_2PO_4 , anhydrous)
- 500.0 c.c. distilled water.

Follow directions for making solution A.

If a scum appears on either of these two solutions, filter again. The same solution may be used for weeks without deterioration. The eosin solution should be removed when a greenish color appears which is due to the carrying-over of the methylene blue. Keep the stain in covered jars, at a depth of about 3 inches. Maintain this level by adding more stain from time to time.

If azure I* is not obtainable, good results are secured by mixing methylene blue from medicinal methylene blue and a buffer as follows:

Dissolve 1.3 gm. medicinal methylene blue
and 5.0 gm. anhydrous Na_2HPO_4
in 50.0 c.c. distilled water.

Bring to a boil. Evaporate on a water bath almost to dryness.

Add 6.25 gm. anhydrous KH_2PO_4 .

Add 500.0 c.c. distilled water.

Stir until the stain is completely dissolved and set aside for 24 hours.

Filter before use.

Technic of Staining Method.—

Dip into Solution A for 1 second.

Rinse by "waving" in water for a few seconds until no more stain comes off.

Dip into Solution B 1 second.

Rinse by gently "waving" in water for 2 to 3 seconds.

Place vertically in rack to dry.

Staining of parasites is optimal at the lower edge of the film toward which the hemoglobin has drained.

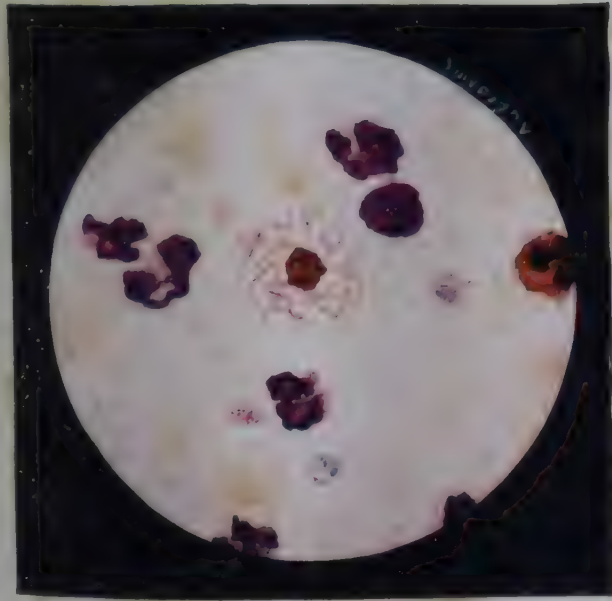
Colors.—

General ground: creamy yellow color, sometimes uniform, sometimes mottled with pale blue.

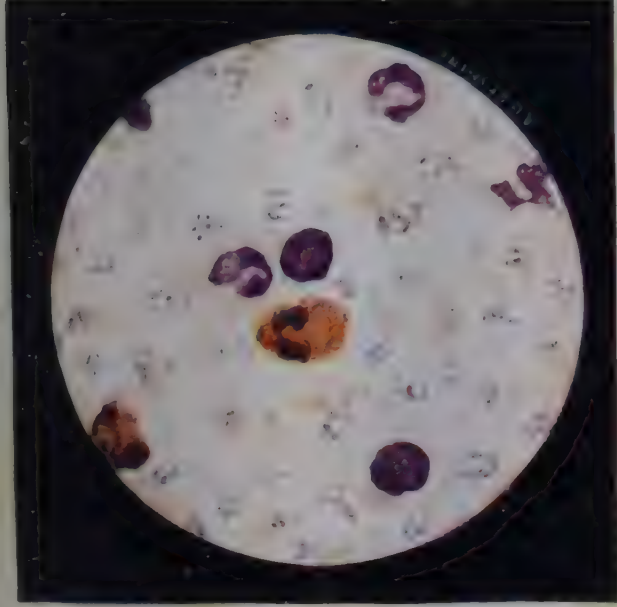
Leukocytes: *Nuclei*—deep blue, sharply defined. *Cytoplasm*—pale blue, vaguely defined. *Granules*—*eosinophilic:* large, red, well defined; *neutrophilic:* small, pale purple, vague.

Malarial Parasites: *Cytoplasm*—blue. *Chromatin*—dark purplish red. *Pigment*—unstained, yellowish of varying shades.

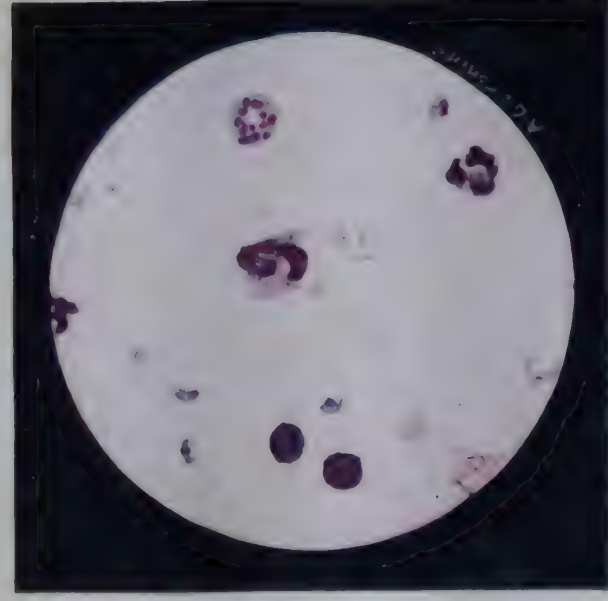
*American equivalent of German azure I is azure B.



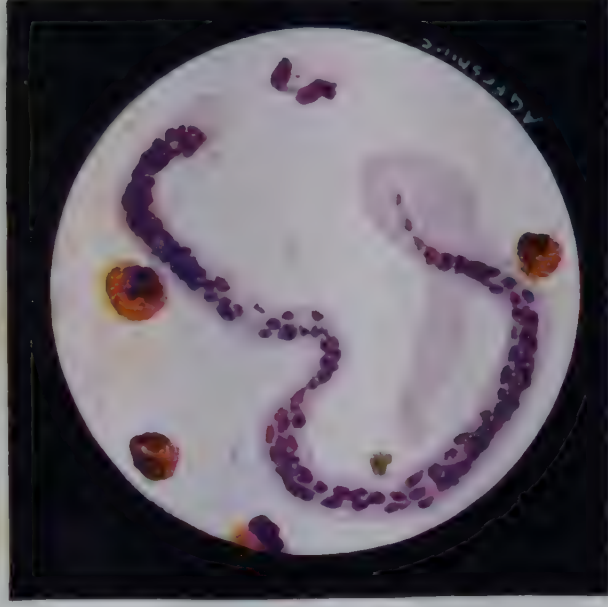
NORMAL THICK DROP
X 950



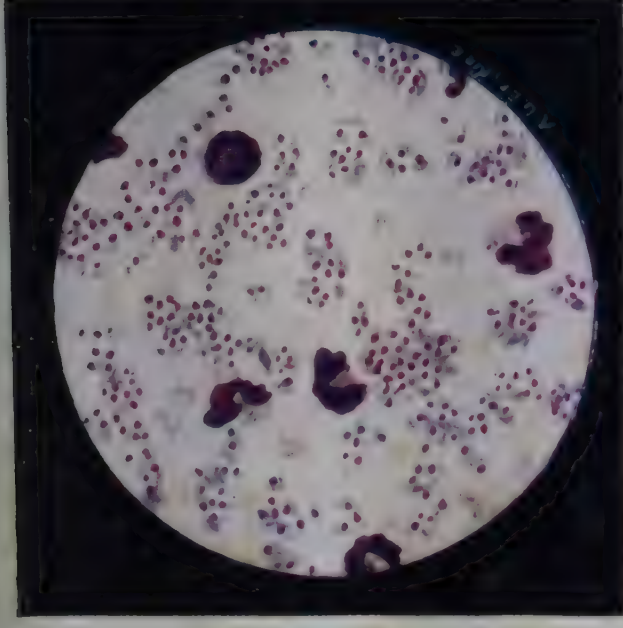
LEAD POISONING
X 950



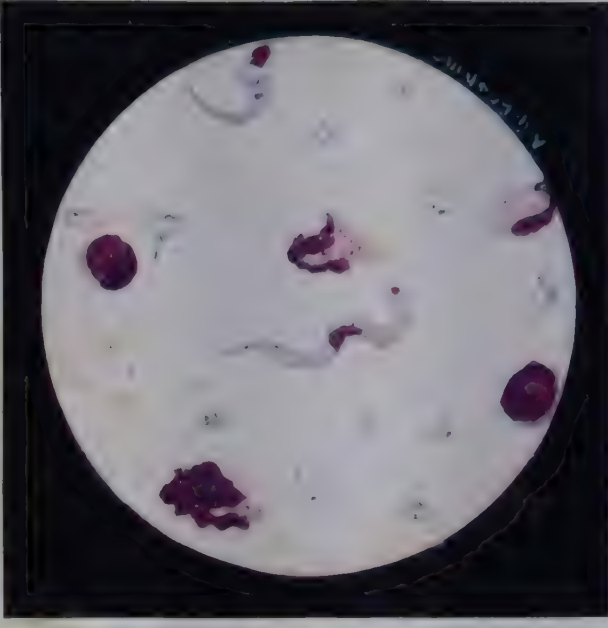
MALARIAL PARASITES
X 950



FILARIASIS BANCROFTI
HEMATOXYLIN-GIEMSA STAIN
PLATE XIII.—THICK DROPS.



PLASMODIUM FALCIPARUM IN
FATAL TROPICAL MALARIA
X 950



TRYPANOSOMIASIS GAMBIENSE
X 950

Field notes that the only disadvantage of this stain is that in anemic bloods there are chromatoid and reticular residues of immature red cells which stain more obtrusively than with the Giemsa stain and may confuse an inexperienced worker.

Examination of the Thick Drop

The thick drop is useful in determining polychromasia, basophilic punctation, eosinophiles, and parasites.

After drying in the air, examine with an oil immersion objective. Polychromasia appears as small net structures stained blue. Basophilic punctation appears as blue dots arranged in a small circle. Orthochromatic erythrocytes are dissolved.

Report polychromasia as follows:

P-	No polychromatic structures in the preparation
P±	Only one or two such structures in each field
P+	3 to 10 such structures on the average in each field
P++	10 to 20 such structures on the average in each field
P+++	Many such structures on the average in each field
P++++	Very many such structures on the average in each field.

Lead poisoning shows +++ or ++++ polychromasia. In regenerative anemias polychromasia depends on the amount of regeneration.

Basophilic punctation may be reported with the same scheme as polychromasia.

Thick Film Method for the Diagnosis of Malaria*

Barber-Komp

1. Slides should be clean and fat-free and should not be fogged or scratched.
2. Make a thick film of blood by placing 4 small drops of blood on a slide and spreading it to about 2 cm. in diameter. Preparations must not be too thin.
3. Allow to dry thoroughly in the air in a horizontal position (1 or 2 hours).
4. In surveys for malarial parasite carriers, hundreds of preparations must be examined. Carry out the technic in groups of 25 slides, as follows:
 - (a) Films must be at least 1 cm. from the end of slides.
 - (b) Labels are placed at the other end of the slides.
 - (c) Place the slides in a box and insert between the labeled ends blocks of cardboard separators $\frac{1}{16}$ to $\frac{1}{8}$ inch thick and $1\frac{1}{4}$ inches long, assemble the slides and cardboard separators, and fasten them together with a stout rubber band. The entire group may be stained as a single unit.
5. Giemsa stain is diluted with neutral distilled water immediately before use. The pH of the water should be between 7.0 and 7.2. Use 1 drop of Giemsa stain for every c.c. of neutral distilled water, shaking the mixture slightly after the addition of each drop. Do not shake too vigorously or a precipitate will form.
6. Immerse the slides in a vertical position in a staining dish containing the diluted Giemsa stain. There should be enough stain to cover the preparations, but it must not interfere with the labels. Stain for 30 minutes to 1 hour, depending on the degree of stain desired. Discard the diluted stain.
7. Rinse gently with neutral distilled water. If the slides are overstained, rinse for 2 to 5 minutes in water.
8. Allow to dry in the air and examine with the oil immersion objective.

*Pub. Health Rep. 44: 1929.

Staining Thick Drops With Wright Stain

Prepare the thick drop in the usual manner and allow to dry thoroughly. Place in a solution of 2% Formalin and 0.2% acetic acid, and let the preparation dehemoglobinize for 2 or 3 minutes. Neutralize in 2% solution of sodium bicarbonate. Stain as usual with Wright stain. After drying thoroughly in the air, examine with the oil immersion objective.

Counting Eosinophiles

Eosinophiles may be counted by the thick drop method.

Count all the white cells and eosinophiles in several fields until 500 leukocytes in all have been counted. The eosinophiles are included in the total. Count the total leukocytes as usual (white count).

The percentage of eosinophiles is the number of eosinophiles in 100 cells. If 500 cells are counted, divide the number of eosinophiles by 5; if 200 are counted, divide by 2, etc. In the example, 500 cells were counted; 25 divided by 5 is 5% eosinophiles. *Normal* is 2 to 4%.

Number of eosinophiles in 1 cu. mm. of blood is the total leukocyte count multiplied by the per cent of eosinophiles. Suppose 25 eosinophiles were counted in 500 cells. Total leukocyte count is 7,500. Number of eosinophiles is $7,500 \times 0.05 = 375$ (the absolute count).

Direct Count of Eosinophiles in the Blood

The usual differential count is sufficient to determine the relative number of eosinophiles in the blood. To find the number per unit volume of blood, the actual count must be made.

Method of Friedman¹

Reagent.—

Aqueous eosin, 1%	5 c.c. ^{2, 3}
Acetone	5 c.c.
Distilled water	100 c.c.

Technic.—

Make the count in the usual manner, in a white blood counting pipette. The diluting fluid stains only the eosinophilic cells, which are easily distinguishable with the high dry power of the microscope. The unstained leukocytes appear as gray bodies in this diluting fluid.

To check the accuracy of the method, fill the pipette with the ordinary acetic acid diluting fluid and proceed to make a count. Check this with the count made with the staining solution.

Figure the relative and absolute counts as discussed on page 655.

Method 2

Reagents.—

Solution I.—

0.1% Methylene blue in propylene glycol	50 c.c.
Distilled water	50 c.c.

Solution II.—

0.1% Phloxine in propylene glycol	50 c.c.
Distilled water	50 c.c.

Mix 4 drops of Solution I with 6 drops of Solution II in a watch glass. This mixture is usable for 4 hours.

¹Friedman, T. B.: *J. A. M. A.* **103**: 1618, 1934.

²Schilling, Victor: *The Blood Picture*, translated by Gradwohl, St. Louis, 1929, The C. V. Mosby Co.

³von Domarus: *Deutsches Arch. f. klin. Med.* **171**: 333, 1931.

Technic.—

Draw blood to the mark 1.0 (top of the stem) in a white counting pipette.

Draw the diluting fluid into the pipette until a total of 11 volumes is reached. The dilution of the blood is 1:10.

Shake well.

Let stand for 10 to 15 minutes.

Again shake well, let out a few drops, and fill both sides of the counting chamber as usual.

Let stand in the counting chamber for 3 minutes.

Count under the low power with the condenser in place. Adjust the light by *slightly* lowering the condenser until the maximum number of white cells is seen and the eosinophiles appear as very tiny red stained cells. The eosinophilic granules are brilliant red.

Count all the white cells, including the eosinophiles, in the four corner fields.

Divide the sum by 4, and multiply by 100 (instead of the usual 200 because of the difference in the dilution) to obtain the patient's white blood count.

Count 2 drops and take the average.

Count all the eosinophiles in the entire 9 fields of the counting chamber (9 sq. mm.).

Multiply the sum in the 9 fields by 11 to obtain the number of eosinophiles in 1 cu.mm. of blood. (Dilution of blood is 1:10, area counted is 9 sq. mm., depth of the counting chamber is 1/10 mm. The volume counted is $1/10 \times 9 \times 1/10 = 9/100$, or approximately 1/11 cu.mm.).

To Obtain the Per Cent of Eosinophiles.—

The per cent of eosinophiles is the eosinophile count per cu. mm. divided by the white blood cell count per cu.mm.

Report the count made in the counting chamber as the absolute count and the percentage as indicated.

Example.—

Patient's white count: 8,163 per cu.mm.

Eosinophiles: 276 per cu.mm.

$276 \div 8,163 = 0.03$, or 3%.

Normal.—

2 to 4%; 150 to 300 per cu.mm., average 200. **Note:** The 0.1% methylene blue and the 0.1% phloxine in propylene glycol are stock solutions and may be diluted before use, or prepared and kept about one month. If they precipitate, filter.

Thorn Test

The blood eosinophile response to the injection of pituitary adrenocorticotrophic hormone (ACTH) or epinephrine is the basis of the Thorn test¹ of adrenal cortex function. In normal subjects, such injections are followed in 4 hours by a fall in the total blood eosinophile count of over 50%. In patients with Addison's disease, the decrease in eosinophiles is 20% or less. In patients with adrenal insufficiency secondary to pituitary disease, the injection of epinephrine is followed by no significant change in the eosinophile count, but injection of ACTH produces a normal decline in eosinophiles.

This screening procedure is performed with the patient starting in the fasting state and remaining so throughout the 4-hour period of observation. A total blood eosinophile count is performed and then 25 mg. of ACTH or 0.3 c.c. of a 1:1,000 dilution of epinephrine is injected intramuscularly. Four hours later another eosinophile count is performed and this value is compared with the original count.

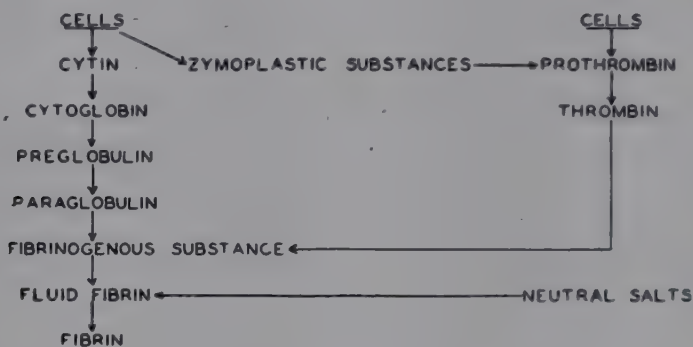
¹Thorn, G. W., Forsham, P. H., Prunty, F. T. G., and Hills, A. G.: J. A. M. A. 137: 1005, 1948.

COAGULATION OF BLOOD

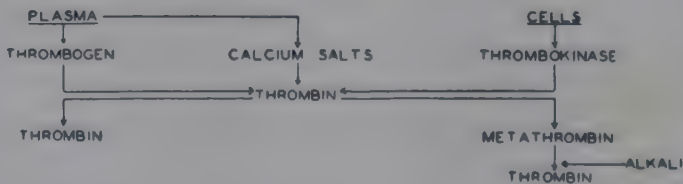
Theories of Blood Coagulation and the Laboratory Aspects of Hemostasis

Blood clotting has been the subject of exhaustive study for many years. Knowledge of the theories of blood coagulation is helpful when, having ascertained from a complete history and physical examination that some bleeding diathesis exists, the physician must pin point the disorder and select the proper laboratory tests to be made.

The ferment theory was first advanced by Alexander Schmidt.¹ He emphasized that the specific ferment for blood coagulation was thrombin, which was a product which occurred with the breaking down of the corpuscular elements of the blood, especially the leukocytes. A schematic representation of the Schmidt ferment theory follows:

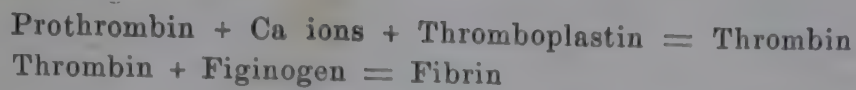


It was Hammarsten² who introduced the question of the role of fibrinogen into blood coagulation. Arthus and Pagès³ first called attention to the calcium content of the blood as of prime importance in blood clotting. They noted that the addition of oxalates to blood, which made it calcium-free, prevented clotting and that the re-addition of calcium produced clotting. Pekelharing⁴ made further researches into this calcium factor. Hammarsten claimed that the presence of calcium is necessary before prothrombin could be activated into thrombin. Fuld⁵ and Spiro⁶ showed that three substances, which are precursors of thrombin, are necessary for blood clotting: namely, the thrombogen of Morawitz, the plasmochyme of Fuld, and soluble calcium salts. Another substance which Morawitz introduced into the literature was thrombokinase, which Fuld called cytozyme. The following diagram illustrates Morawitz' ideas on blood clotting.⁷



¹Schmidt, Alexander: *Zur Blutlehre*, Leipzig, 1892; *Weitere Beitrage zur Blutlehre*, Wiesbaden, 1895.
²Hammarsten: *Pflueger's A.* Bd. 30.
³J. de Physiol. 22/1890.
⁴Pekelharing: *Zentralbl. f. Phys.* 9/1895.
⁵Fuld: *Zentralbl. f. Phys.* 9/1895.
⁶Spiro: *Hofmeisters Beitr.* 5/1903.
⁷From Hittmair: *Handbuch der allgemeinen Haematologie*, Bd. II, Berlin, 1934, Urban and Schwarzenberg.

Howell⁷ is responsible for the present-day understanding of the phenomena of blood coagulation.



Within the past fifteen years, interest in blood coagulation has been greatly stimulated, and as a result a voluminous literature concerning hemostasis has been accumulated. As new facts have been disclosed, theories of blood coagulation have been modified and amplified. Figs. 171 to 175 show the concepts of outstanding coagulationists, summarizing leading thoughts on the problem.

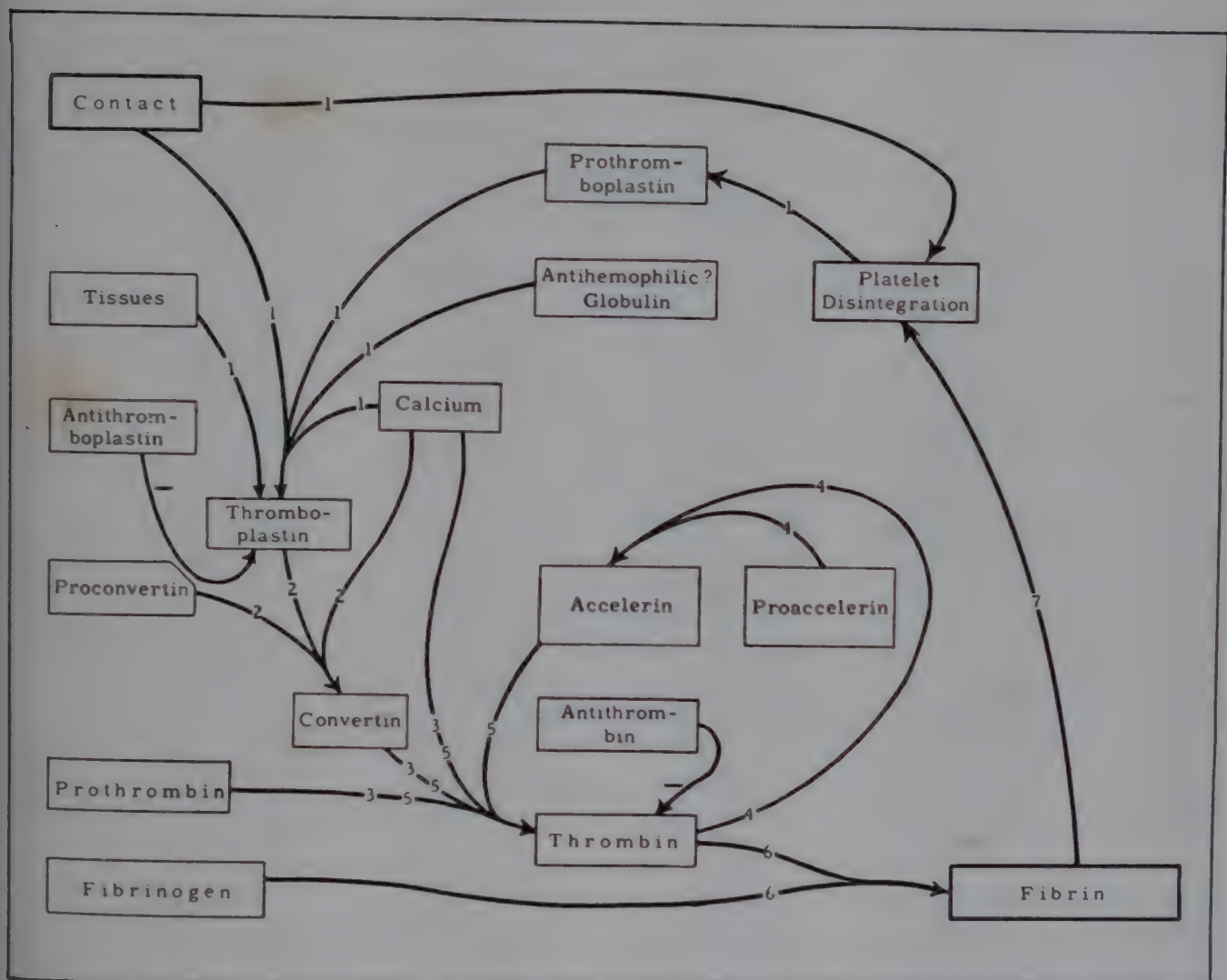


Fig. 171.—Blood coagulation, theory of P. A. Owren (1952). 1, Tissue injury yields thromboplastin directly, while contact causes disintegration of platelets and release of prothromboplastin, which is activated by contact, "antihemophilic globulin" and calcium to give thromboplastin. 2, Thromboplastin and proconvertin in the presence of calcium form convertin. An anticonvertin probably exists which opposes the activity of convertin. 3, Convertin together with calcium brings about a minimal conversion of prothrombin to thrombin. 4, This initially formed thrombin starts the accelerator system, i.e., the conversion of proaccelerin to accelerin. 5, Accelerin accelerates the conversion of prothrombin to thrombin in the presence of convertin and calcium. 6, Thrombin is now in sufficient quantity to convert fibrinogen to fibrin. 7, Fibrin provokes the disintegration of the platelets with further release of thromboplastic substances already mentioned. (From Albritton, E. C.: *Standard Values in Blood*, W. B. Saunders Co.)

According to the **theory of Owren**, thromboplastin is made available from two sources, directly from tissue juices and indirectly from platelet disintegration. Platelet disintegration, resulting from contact with foreign sur-

⁷Howell, W. H.: *Bull. Johns Hopkins Hosp.* 42: 119; *Am. J. Physiol.* 63: 434; 71: 553; 77: 670; *J. A. M. A.* 117: 13, 1059, 1941.

faces, produces prothromboplastin. This precursor form is then converted to thromboplastin by the action of calcium and possibly antihemophilic globulin. Thromboplastin and calcium activate proconvertin to convertin. The proconvertin-convertin complex is considered "stable component" by Stefanini. Convertin and calcium initiate the conversion of prothrombin to thrombin. However, the reaction is suboptimal for the interaction of fibrinogen and thrombin to form fibrin. At this stage, though, sufficient thrombin has been formed to convert proaccelerin to accelerin ("serum accelerator"). From this point on, the

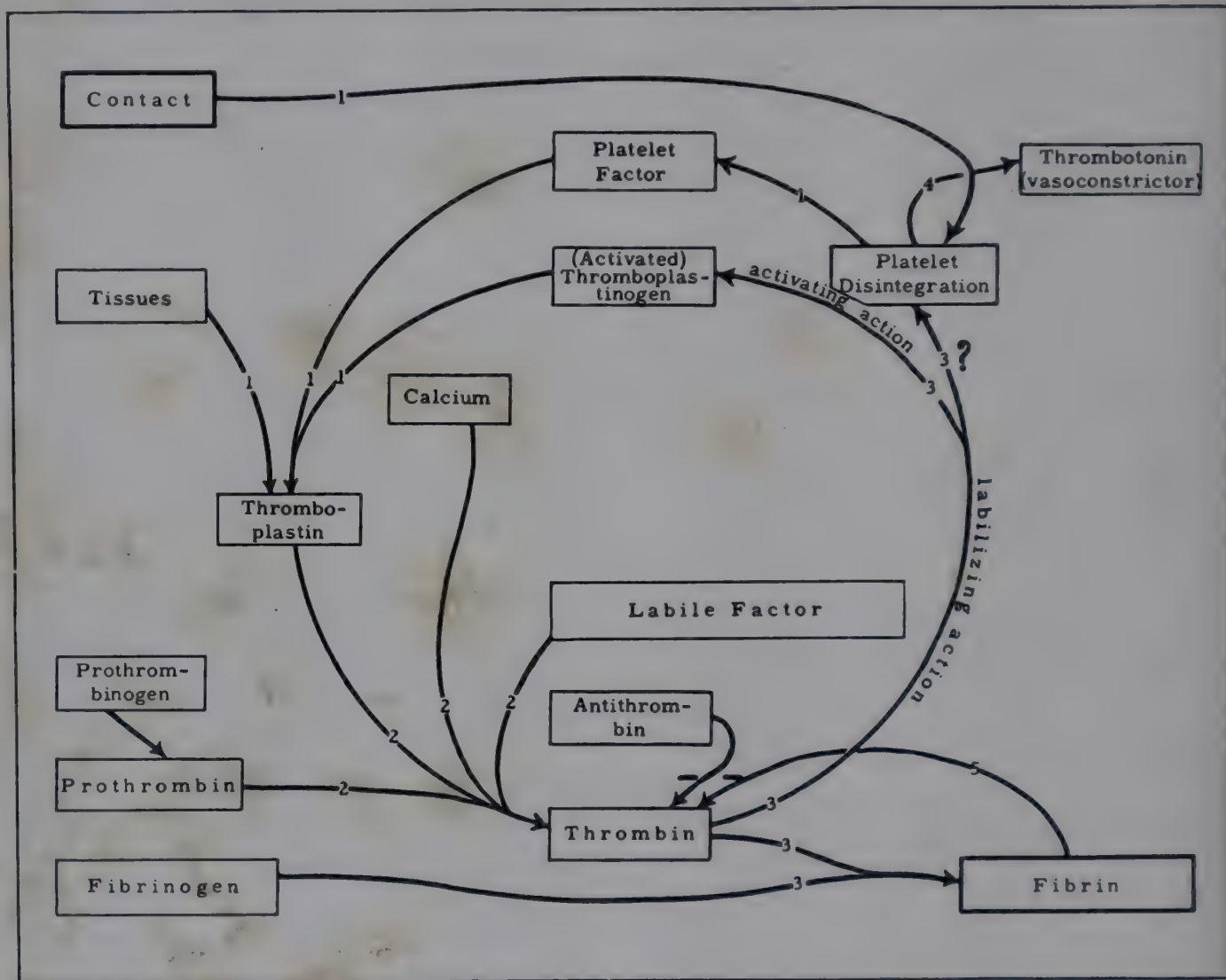


Fig. 172.—Blood coagulation, theory of A. J. Quick (1952). 1, Thromboplastin is (a) released directly by injured tissue and is also (b) formed by the interaction of plasma thromboplastinogen and a platelet factor, the latter released from disintegrating platelets. 2, Thromboplastin, prothrombin, calcium, and a labile factor interact stoichiometrically to form thrombin. In human blood part of the prothrombin is in an inactive form, prothrombinogen. On contact with a rough surface it becomes activated. 3, The thrombin formed not only converts fibrinogen to fibrin, but activates thromboplastinogen and thereby probably brings about the lysis of platelets and initiates the chain reaction. 4, The platelets also release a vasoconstrictor, thrombotonin, under the labilizing action of thrombin. The resulting local vasoconstriction aids in the hemostatic process. 5, The prompt removal of thrombin by fibrin holds in check the autocatalytic reaction mediated through the action of thrombin on platelets and thromboplastinogen. (From Albritton, E. C.: *Standard Values in Blood*, W. B. Saunders Co.)

entire reaction gains momentum, the accelerin-calcium-convertin system converting prothrombin to thrombin in sufficient quantities for the thrombin-fibrinogen reaction to form fibrin. Owren does not visualize fibrin formation initially as an inert end stage. On the contrary, fibrin acts as a foreign surface, resulting in further platelet disintegration and intensification of the total reaction.

The **theory of Quick** visualizes a three-stage coagulation mechanism. In stage 1, thromboplastin formation stems from two sources, tissue juice directly and platelets indirectly. Platelet disintegration by virtue of contact with a foreign surface permits the release of thromboplastinogenase, which activates the conversion of a plasma factor, thromboplastinogen, to form thromboplastin. Phase 2 consists of the interaction of thromboplastin, labile factor, calcium, and prothrombin to form thrombin. This reaction, according to Quick, is stoichiometric. A possible precursor form (prothrombinogen) is

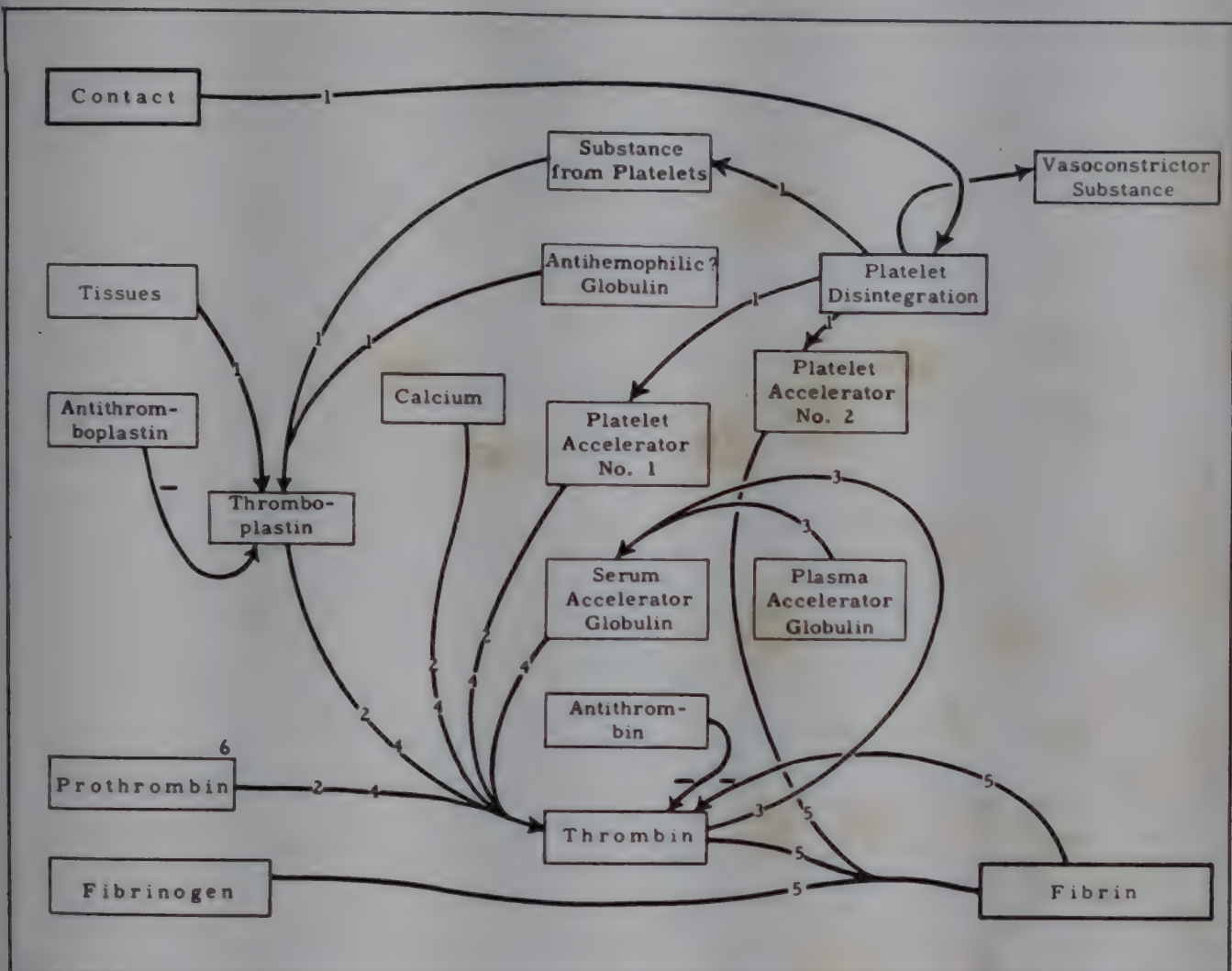


Fig. 173.—Blood coagulation, theory of W. H. Seegers (1952). 1, Injured tissue yields thromboplastin directly, while contact causes disintegration of the platelets and the release from them of (a) a substance which reacts with a globulin in the plasma to give thromboplastin, and (b) two platelet accelerator principles, Nos. 1 and 2. 2, Thromboplastin and calcium, together with platelet accelerator No. 1, bring about a minimal conversion of prothrombin to thrombin. 3, This initially formed thrombin starts the accelerator system, i.e., the conversion of the proenzyme, plasma Ac-globulin, into the enzyme, serum Ac-globulin. 4, Thromboplastin and calcium, together with serum Ac-globulin and platelet accelerator No. 1, cause accelerated prothrombin conversion to thrombin. 5, A now sufficient quantity of thrombin, together with platelet accelerator No. 2, converts fibrinogen to fibrin. Fibrin, by adsorption, inactivates some of the thrombin. 6, Since purified prothrombin can be activated to thrombin by simply placing it in 25 per cent sodium citrate solution, it is believed that prothrombin itself contains all of the necessary material substance for the formation of thrombin. Consequently, the activators of prothrombin are catalysts and do not enter into stoichiometric combination with prothrombin to form thrombin. (From Albritton, E. C.: *Standard Values in Blood*, W. B. Saunders Co.)

simultaneously converted to prothrombin by contact alone. Thrombin formation initiates stage 3, which consists chiefly of the conversion of fibrinogen to fibrin. At the same time the powerful enzyme, thrombin, by its action, labilizes platelet disintegration, resulting in further thromboplastinogenase formation and augmentation of phases 1 and 2. As a brake on the somewhat self-perpetuating process, fibrin adsorbs thrombin and helps bring the action to a close.

The **theory of Seegers** begins stage 1 with thromboplastin formation from tissue juices directly and platelet disintegration indirectly. Platelet disintegration produces three factors, one of which combines with anti-hemophilic globulin to form thromboplastin. The other two factors, platelet accelerator 1 and platelet accelerator 2, play their roles later in the clotting mechanism. Phase 2 consists of the interreaction of thromboplastin, calcium, platelet accelerator 1, and prothrombin with minimal conversion to thrombin.

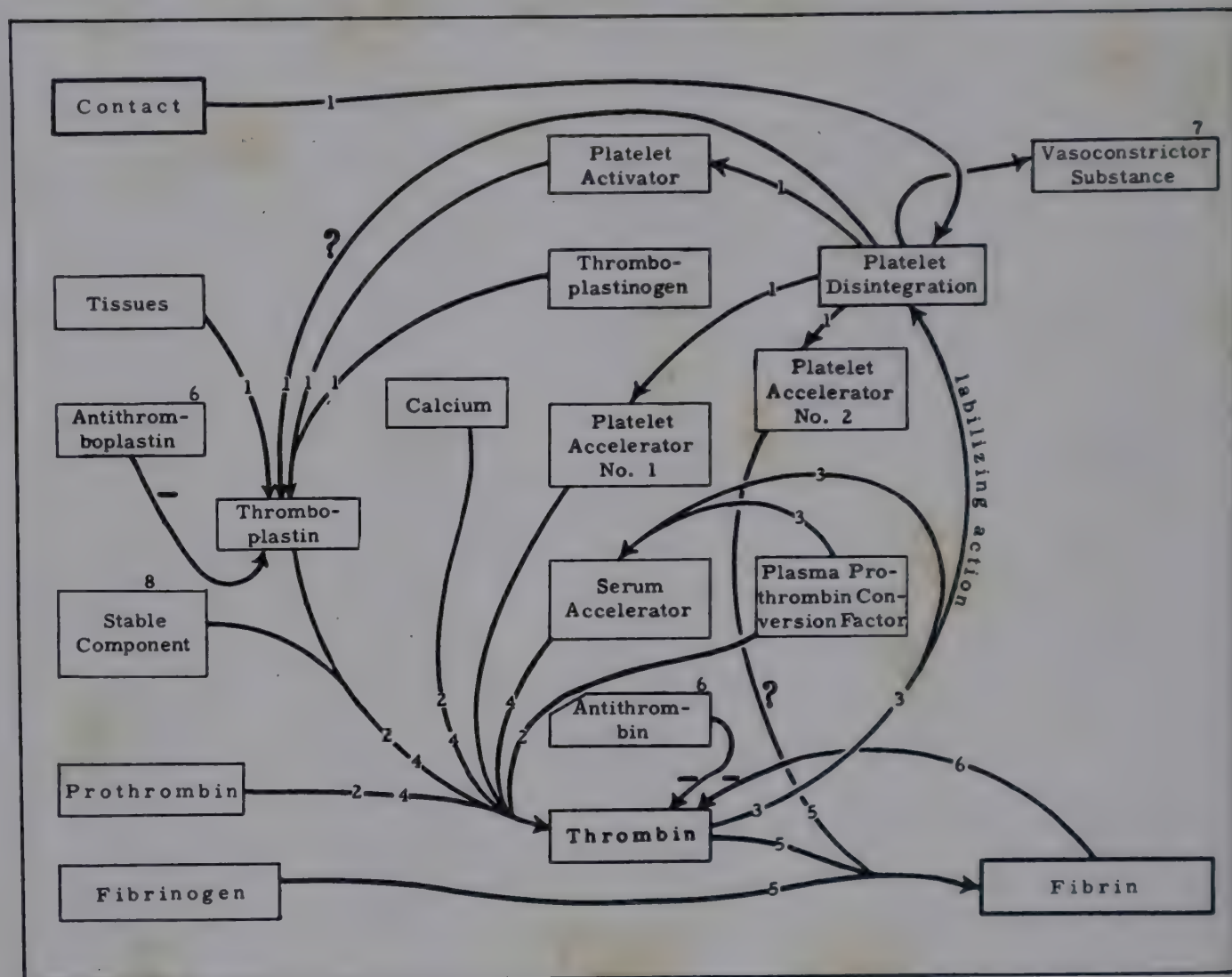


Fig. 174.—Blood coagulation, theory of M. Stefanini (1952). 1, Injured tissue yields thromboplastin directly, while platelet disintegration yields (a) platelet activator, which interacts with plasma thromboplastinogen (and possibly another plasma component) to form thromboplastin, (b) possibly, thromboplastin directly and (c) two accelerator principles, Nos. 1 and 2. 2, Thromboplastin, calcium, plasma prothrombin conversion factor and stable component interact to bring about a minimal conversion of prothrombin to thrombin. 3, This initially formed thrombin initiates the autocatalytic phase of the process, i.e., (a) starts the accelerator system—the conversion of plasma prothrombin conversion factor into a more active form, i.e., serum accelerator, and (b) “labilizes” the platelets to release more platelet activator and accelerators. 4, Thromboplastin and calcium, together with serum accelerator, platelet accelerator No. 1 and, possibly, stable component cause accelerated conversion of prothrombin to thrombin. 5, A now sufficient quantity of thrombin, aided by the action of platelet accelerator No. 2, converts fibrinogen to fibrin. 6, Antithromboplastin and antithrombin act to decrease the speed of coagulation reactions in which they are involved. The fibrin clot, by adsorbing thrombin, also effectively checks the autocatalytic mechanism of blood coagulation. 7, The platelet disintegration which takes place at the site of vascular injury also liberates a vasoconstrictor agent which aids in the arrest of the hemorrhage. 8, Stable component is a factor which appears decreased during Dicumarol therapy and is sharply decreased in the hypoprothrombinemia of the newborn. Its properties are very similar to those of prothrombin. (From Albritton, E. C.: *Standard Values in Blood*, W. B. Saunders Co.)

However, sufficient thrombin formation occurs to potentiate an accelerator system—Plasma Ac-globulin \rightarrow Serum Ac-globulin. Stage 2 is now accelerated sufficiently for pushing through the thrombin-fibrinogen reaction to fibrin formation, with the aid of platelet accelerator 2. Fibrin then acts as a brake by adsorbing thrombin on its surface to bring the process to a close.

Tocantins believes that the initial stage of clotting produces thromboplastin from two sources: tissue juices and platelet disintegration. In this platelet disintegration he postulates the production of two derivatives, platelet accelerators 1 and 2. Platelet factor 1 or lipid thromboplastin produces thromboplastin. From here on the mechanism follows that of Seegers with the introduction of an activated accelerator system to produce optimal fibrin formation. Fibrin serves as a brake on thrombin formation by adsorption and simultaneously acts as a foreign surface for further platelet disintegration.

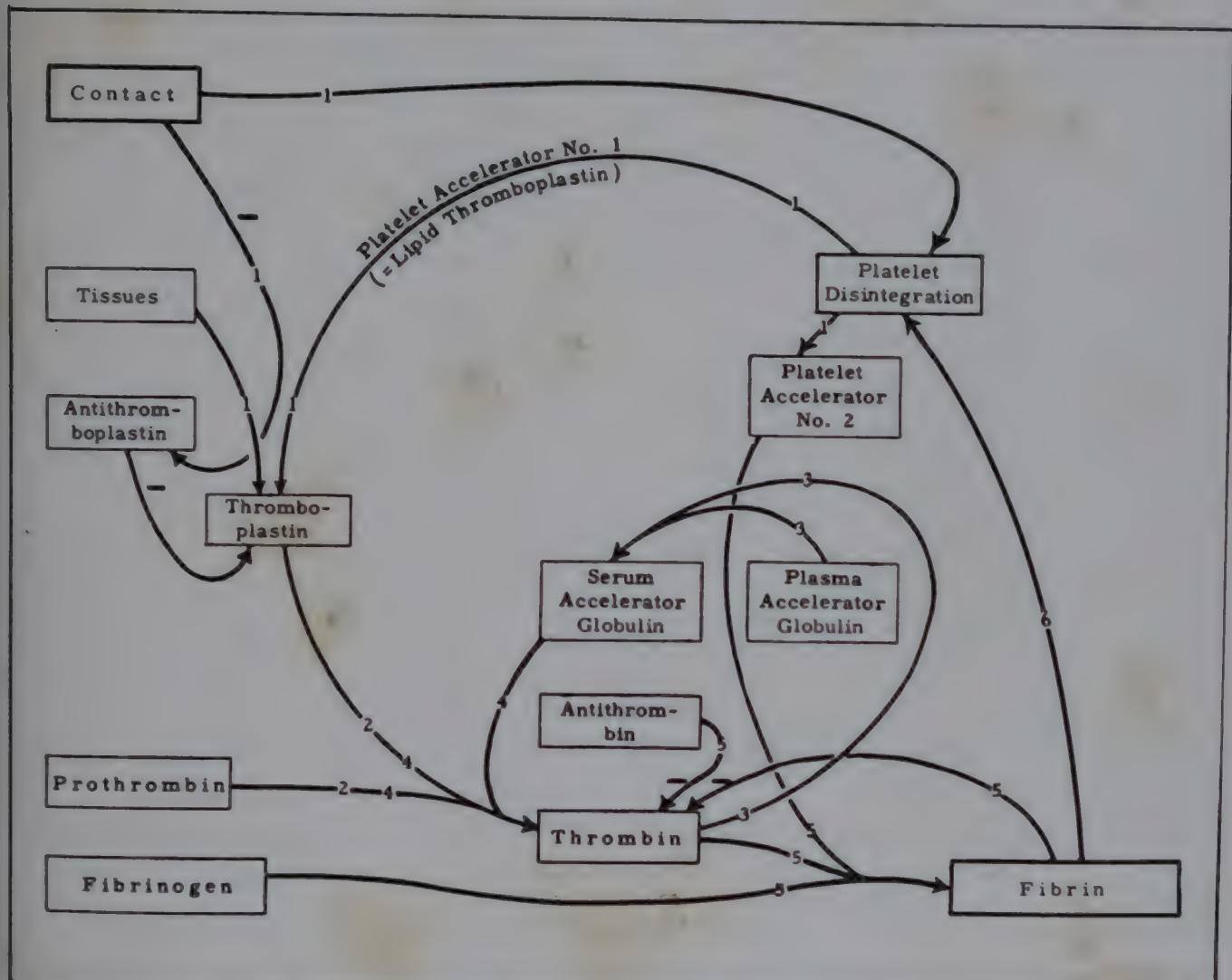


Fig. 175.—Blood coagulation, theory of L. M. Tocantins (1952). 1, Contact of the blood with certain surfaces (damaged blood vessel endothelium, glass) initiates the first changes which lead to the inception of clotting; blood platelets agglutinate, adhere to the surface and/or disintegrate, releasing (a) thromboplastin and (b) fibrinolytic factor (platelet accelerator No. 2). Some of the thromboplastin of the blood is offset or neutralized by the antithromboplastin; some of the antithromboplastin is, itself, adsorbed or neutralized at the contacting surface. 2, Thromboplastin (from tissues and/or platelets) brings about a minimal conversion of prothrombin to thrombin. 3, This initially formed thrombin activates the accelerator system, that is, the conversion of inactive plasma accelerator globulin to active serum accelerator globulin. 4, Thromboplastin, together with the serum accelerator globulin, causes acceleration of the conversion of prothrombin to thrombin. 5, Some of the thrombin may be inactivated by antithrombin. The thrombin that escapes such inactivation acts, with the aid of platelet accelerator No. 2, to cause the conversion of fibrinogen to fibrin. Some of the excess thrombin is removed from the plasma by adsorption on fibrin. 6, Fibrin probably causes further disintegration of platelets. (From Albritton, E. C.: *Standard Values in Blood*, W. B. Saunders Co.)

The **theory of Stefanini** begins with thromboplastin formation from platelets and tissue juice resulting from contact. Platelet disintegration results in three factors: an activator (platelet thromboplastic factor) and two platelet accelerators. The activator converts a plasma component (thromboplastinogen or plasma thromboplastic factor) to thrombin. Stefanini agrees that thromboplastin may be released by platelets directly but this is in minimal

quantities. Thromboplastin, calcium, stable component, and labile component (plasma prothrombin conversion factor) react with prothrombin, resulting in minimal conversion to thrombin. Once thrombin has been formed, labile component is transformed to "serum accelerator," which potentiates the speed and quantity of thrombin formation to a degree sufficient for the conversion of fibrinogen into fibrin. In this latter phase, a platelet accelerator plays a role. Thrombin exerts a labilizing influence on platelets, producing agglutination and lysis with resultant reinforcement of the chain reaction of coagulation. Fibrin applies the brakes to the action by adsorption of thrombin.

Although disagreements as to all phases of coagulation are apparent from the preceding presentations, it is readily apparent that there is much basic accord. All authors agree that the process is a complex mechanism which is still not completely understood. They further agree that platelets play a most important role in initiating the process and activating thromboplastin. Prothrombin conversion to thrombin requires one or more factors other than calcium and thromboplastin for yields sufficiently great to get the mass action necessary for ultimate conversion of fibrinogen to fibrin. There is agreement that fibrin is not an inactive end stage in the coagulation but that it plays an important part in checking the action of thrombin while at the same time promoting further platelet disintegration. The presentation of the theories of coagulation in cyclical form contained herein suggest not only the multiplicity of factors involved but an action not unlike that of a pinwheel in a Fourth of July fireworks display. There is the slow starting outer phase (thromboplastin to minimal prothrombin conversion), a sputter (thrombin formation), a high-speed brilliant phase with sparks flying in all directions (thrombin activating accelerators, thrombin labilizing platelets, more prothrombin being converted, fibrinogen being converted to fibrin), and then the slowly dying-out phase (a firm clot being formed and fibrin slowly applying the brakes).

Thus far only the more prominent components in blood coagulation have been mentioned. However, there are other controls of hemostasis, perhaps less well understood, which play a role in the dynamics of blood clotting. An important defense mechanism is the release of a vasoconstrictor substance by disintegrating platelets to aid in stopping the flow of blood. Antagonists in the blood stream in the form of antithrombin and possibly antithromboplastin play roles in maintaining the balance necessary for normality or aiding defense.

Another complex related to elements in the clotting mechanism is the fibrinolytic system. There is question as to whether this system plays a role in normal fibrinogen content or in the active phases of coagulation. The same doubt exists as to its role in the disposal of fibrin after a clot is formed. Under recently recognized pathologic conditions, the excess production results in hemorrhagic diatheses. Perhaps the most dramatic of these clinical states is the severe bleeding tendency occurring with premature separation of the placenta, resulting in an apparently complete breakdown in the coagulation mechanism: shock, extensive hemorrhage, severe trauma, surgery (especially of the lungs), leukemia, carcinoma (especially metastatic cancer of the prostate), and chronic liver disease.

Still another group possessing anticoagulants are those cases associated with hyperheparinemia such as occur in persons undergoing intensive radiation therapy with nitrogen mustards, or in the leukemias. Still other anticoagulants of unknown character appear related to forms of isoimmunization.¹

At times abnormal proteins may be factors in abnormal coagulation. The hyperglobulinemia syndrome of Waldenström,² often referred to as "macroglobulinemia" is an instance of such disturbance contributing to hemorrhage. Ultracentrifugation reveals an increased percentage of macroglobulins and a serologic test for the presence of macroglobulins has been developed.³

Much has been made (and written) about the disagreements among coagulationists, which attests to the complexity of the problem. A good peek at the problems (and perhaps personalities) involved can be had by perusal of the Josiah Macy, Jr., Foundation publications, on *Blood Clotting and Allied Problems*. The list of participants and guests includes many whose contributions have played important roles in the modern understanding of the problems of coagulation. In addition to the incomplete knowledge of the mechanisms, there has been created an unduly magnified impression that disagreement is the rule among coagulationists. This impression is enhanced by the multiplicity of terms used by the many observers in this field. Even a committee of the experts offers a list of synonyms with apologies for "incomplete knowledge of the literature."⁴ Stefanini has attempted to list synonyms in the literature with bibliography for the authors of each. (See Table 51.)

Many of the factors postulated in group C are very likely mixtures of "labile component" with prothrombin or, even more probably, of prothrombin with "stable component."

Some of the factors in group C are arranged in couples, joined by an arrow. Those at the left of the arrow are considered precursors and found as such in plasma; those at the right are considered the active (or more active) form and are found in the serum. For this reason, some investigators prefer to think of "labile" and "stable" component as a system.

One of the major points that Stefanini makes about many of these synonymous terms is that they do not necessarily represent specific substances but that they are "systems" of reaction complexes. He points out that this is particularly true in the group related to the conversion of prothrombin to thrombin, an area where there is perhaps the greatest amount of disagreement as to the mechanisms involved. Disagreements here, as elsewhere, are often due to variations in technic. Often the substrates used in laboratory tests or in experimental studies are prepared in different manners or from different sources. As a result, these substrates may be varying mixtures, and tests, superficially similar, in different laboratories may not give reproducible results. This feature is recurrently mentioned in the discussions in the *Proceedings of the Fifth Conference on Blood Clotting and Allied Problems*. The problem related to the Quick one-stage prothrombin determination used

¹Dreskin, O. H., and Rosenthal, N.: *Blood* 5: 46, 1950.

²Waldenström, J.: *Acta. med. scandinav., supp.*, 266: 931, 1952.

³Wilde, H., and Hitzelberger, A. L.: *Blood* 9: 875, 1954.

⁴Subcommittee on Synonymy of the Nonprothrombin Plasma Factors Involved in the conversion of Prothrombin to Thrombin, *Blood Clotting and Allied Problems*, J. E. Flynn, ed., Tr. Fifth Conf. New York, Josiah Macy, Jr., Foundation, 1949; p. 330.

TABLE 51.—SYNONYMS OF VARIOUS FACTORS ACTIVE IN THE COAGULATION OF BLOOD,
DEMONSTRATED IN MAN

<i>A. Factors Involved in the Activation of Thromboplastin</i>	
Platelets thromboplastic factor	thromboplastinogenase cellular thromboplastic component
Plasma thromboplastic factor	prothrombokinas plasmakinin antihemophilic globulin thromboplastinogen thrombocytolysin thrombokatalysin plasma thromboplastic component
<i>B. Thromboplastin (Tissues)</i>	
	thrombokinas cytozime thromboplastic protein thrombokinin
<i>C. Factors Involved in the Conversion of Prothrombin to Thrombin Other Than Calcium and Thromboplastin</i>	
Plasma "labile component"	thrombogène component A of prothrombin factor V → factor VI accelerator factor labile factor co-factor of thromboplastin plasma Ac globulin → serum Ac globulin proprothrombinase → prothrombinase prothrombinogenase → thrombinogenase ? prothrombinokinase → thrombokinas* plasma prothrombin conversion factor → serum accelerator
Plasma "stable component"	proaccelerin → accelerin co-factor V ? component B of prothrombin prothrombin accelerator prothrombin conversion factor prothrombin converting factor co-thromboplastin plasma precursor → serum prothrombin conversion accelerator proconvertin → convertin factor VII ? inactive prothrombin

*Meant to signify any agent capable of influencing the conversion of prothrombin to thrombin.

From Stefanini, M.: J. Mt. Sinai Hosp. 19: 619, 1953. Reproduced by the courtesy of the author and editor.

routinely in most laboratories is a well-known one. Here one of the basic requisites for consistently good results has been a thromboplastin of regular activity.

This problem is relatively simple compared with studies of more complex mechanisms. Stefanini¹ and Owren² have both outlined procedures for the determination of prothrombin and accessory factors. Basically, these tests consist of the additions of various reagents to either remove or add factors to a sample of plasma with resultant constancy of other factors. At times, these workers and others in the field have had to use the blood of patients or animals with deficiency diseases related to these factors as substrates.

¹Stefanini, M.: Am. J. Med. 14: 64, 1953.

²Owren, P. A.: Am. J. Med. 14: 201, 1953.

Another approach to the problem has been correction experiments in which the bloods in various deficiencies have been cross-mixed to prove the similarity or dissimilarity of disease processes. This technic has been a very common tool in the study of hemophilia and hemophilia-like diseases.^{3, 4} The deficiency diseases, themselves, have been the source of much of the study of basic concepts in coagulation.⁵

In summary it might well be reiterated that the intricate and complex mechanism of blood coagulation has been a subject of intense investigation with many problems unsolved. These investigations have not only led to a better understanding of blood clotting but have revealed new disease entities (such as the hemophilia-like diseases) and have resulted in useful tools in the management of disease (Dicumarol and others).

TESTS IN THE HEMORRHAGIC DISEASES

I. Tests for the Status of Capillaries

(A) BLEEDING TIME OF THE SKIN

Method I, Duke⁶

Normal Values: 1 to 3 minutes.

Technic.—

Cleanse the ear lobe and make a cut, not a puncture, with a scalpel blade, needle, or Steri-Lancet, 3 to 4 mm. deep and approximately 5 mm. long.

Wipe the wound area once with dry sterile cotton.

Touch a piece of blotting paper to the area every 30 seconds, touching only the head of the drop. The test is terminated when no more free drops can be picked up on the blotting paper.

The bleeding time is measured from the moment of the incision to the cessation of bleeding. Multiply the number of spots of blood by 30 (seconds) to obtain the bleeding time.

Method II, Ivy⁷

Normal Values: 0 to 4 minutes.⁸

Materials Required.—

Sphygmomanometer.

Sharp-pointed blade.

Technic.—

Place a blood pressure cuff on the arm and inflate to 40 mm. of mercury.

Cleanse the volar surface of the forearm below the cuff, and make a skin wound 3 to 4 mm. deep.

Blot the incision every 10 seconds until there are no more free drops, maintaining a pressure of 40 mm. throughout the procedure.

The bleeding time is measured from the moment of the incision to the cessation of bleeding. Count the blood spots and multiply by 10 (seconds).

³Brinkhous, K. M., Langdall, R. D., Penick, G. D., Graham, J. B., and Wagner, R. H.: *J. A. M. A.* 154: 481, 1954.

⁴Rosenthal, R. L.: *Am. J. Med.* 17: 57, 1954.

⁵Alexander, A., Goldstein, R., Rich, L., Le Bolloc'h, A. G., Diamond, K., and Borges, W.: *Blood* 9: 843, 1954.

⁶Duke, W. W.: *J. A. M. A.* 55: 1185, 1910.

⁷Ivy, A. C., Shapiro, P. F., and Melnick, P.: *Surg., Gynec. & Obst.* 60: 781, 1935.

⁸Ivy, A. C., Nelson, D., and Bucher, G. R.: *J. Lab. & Clin. Med.* 26: 1812, 1941.

Method III, Copley and Lalitch⁹

Normal Values: In 95% of persons, the normal values are less than 3 minutes with a range of 170 to 340 seconds.

Technic.—

Cleanse the finger and make a puncture wound 6 mm. deep.

Immerse the wound in sterile physiologic saline warmed to 37° C. until there is no free flow of blood.

The bleeding time is measured from the moment of the wound to the cessation of bleeding.

Comments on the Skin Bleeding Time.—

The response of the skin capillary bed as measured by these tests is dependent upon many factors, such as skin turgor, adiposity and vascularity of subcutaneous tissue, variations in technic, as well as the state of the blood vessels and the blood coagulation mechanism. Obviously, any test which attempts to measure so many variables can give information only of a general nature and serves only as a gross screening test.

Abnormally prolonged bleeding times are associated with thrombopenic states, whether they are primary or secondary.

In a discussion on the mechanism of hemostasis, Macfarlane¹⁰ called attention to the lack of capillary contractility associated with thrombocytopenic purpura. By visualization of the nail beds under the microscope, he noted differences in the capillary reaction in various conditions and particularly called attention to the abnormal reaction in thrombocytopenia. This reaction of capillaries is of such marked degree that it might be considered an integral part of the disease process. It is our impression that the extent of bleeding manifestation in cases of thrombocytopenic purpura is as directly related to the state of the capillary bed as it is to the platelet level. Although bleeding times and platelet levels usually parallel one another, there are cases in which the bleeding time may be prolonged with only a slight reduction in platelets and in other cases the reverse may be true. It has been suggested that an antigenic relationship exists between platelets and vascular endothelium with a common etiology for both.¹¹

Another defect in clotting mechanism associated with prolonged bleeding time is hypoprothrombinemia. The experience of surgeons prior to the discovery of the value of vitamin K therapy amply attests to the capillary bleeding in patients with obstructive jaundice.

A surprising relationship between a severe abnormality in the coagulation mechanism and a normal bleeding time test exists in hemophilia. With the minor wound inflicted in the test, the capillaries are sufficiently resilient to shut off immediate bleeding. However, it is not at all uncommon for bleeding to return at the puncture site an hour or more later. Occasionally such recurrent bleeding may be sufficiently severe to cause concern. If hemophilia is strongly suspected, laceration of the skin should be zealously avoided, and punctures for tests should be limited only to those necessary for diagnosis and

⁹Copley, A. L. and Lalitch, J. J.: *J. Clin. Invest.* 21: 145, 1942.

¹⁰Macfarlane, R. G.: *Quart. J. Med.* 10: 1, 1941.

¹¹Ackroyd, J. F.: *Am. J. Med.* 14: 605, 1953.

treatment. The bleeding time is an unnecessary test in this condition. In other diseases simulating hemophilia, such as deficiency of plasma thromboplastin factors PTC (plasma thromboplastin component) and PTA (plasma thromboplastin antecedent), Rosenthal¹² reported usually normal bleeding times.

There exists a nondescript group of hemorrhagic diatheses, most of which have appeared in the older literature, associated with a prolonged bleeding time. Among these might be mentioned the hereditary capillary fragility group, variously labeled pseudohemophilia, von Willebrand's disease, and purpura of capillary weakness. Glanzmann's thrombasthenia is another type in which some unknown defect of platelets, which are normal in numbers, is reputedly responsible for abnormal bleeding. As our knowledge of the clotting mechanism is elaborated, this group becomes more circumscribed.

Bleeding time may be prolonged in elderly individuals in whom no defect in clotting mechanism exists. Such abnormal bleeding is purportedly related to lack of elasticity of capillaries in the aged.

Another paradoxical relationship exists in which purpura (nonthrombocytopenic) is pronounced, capillary fragility tests are markedly abnormal, and yet the bleeding time tests are usually normal. Some of the allergic purpuras fit into this category as do those related to sepsis, uremia, and vitamin deficiencies.

(B) CAPILLARY FRAGILITY TESTS

Capillary fragility tests can be roughly divided into two principal groups, those applying pressure and those applying suction. Many modifications, chiefly aimed at standardization of the technic, have been devised.

Method I, Rumpel-Leede¹³

This test, which originally consisted in placing a tourniquet about the arm and observing the number of petechiae in the area distal to it, has had so many modifications of time of application and levels of pressure that the authorships of most of the modifications can hardly be traced. In general, the tourniquet has been replaced by a blood pressure cuff. There is no agreement as to whether the blood pressure should be midway between diastolic and systolic pressures or whether 100 mm. of mercury is the desirable level. The time of application is variably suggested as 5 to 10 minutes. There is some difference as to whether the entire extremity should be examined for petechiae or whether the number in a limited area should be counted.

There is also no unanimity of opinion concerning normal values. In general, it can be said that most normal individuals show relatively few petechiae, perhaps 10 or less. Those with a marked capillary fragility show many petechiae, sometimes almost covering the entire skin. We should like to add a few comments based on our own experience with these modifications.

It is not at all uncommon for a band of petechiae to appear at the lower edge of a tourniquet or blood pressure cuff, where the skin has been pinched. We suggest, therefore, that this area be disregarded in an appraisal of the results. Another observation is that it is not at all uncommon for petechiae

¹²Rosenthal, R. L.: *Am. J. Med.* 17: 57, 1954.

¹³Leede, C.: *München. med. Wchnschr.* 58: 293, 1911.

to appear several minutes after the application of pressure or suction. For this reason, our routine procedure in any capillary fragility test is to include a re-examination of the area at least 30 minutes after the application of pressure or suction.

Method II, Gothlin¹⁴

Normal Value: 0 to 12 petechiae (Gothlin index).

Carefully inspect the forearms for petechiae or other marks which might be mistaken for them.

Mark off in ink an area 6 cm. in diameter in the antecubital fossae on each upper extremity.

Apply blood pressure cuffs to both arms and inflate simultaneously to 35 mm. of mercury, maintaining this pressure for 15 minutes with the subject resting his forearms on a table.

Release the pressure and inspect the outlined area for petechiae, using a magnifier and bright light.

Multiply the number of petechiae in both arms by 2.

If the Gothlin index is 8 or less, the result is considered normal; if more than 12, capillary fragility is considered abnormal. If the value falls between 8 and 12, the results are equivocal and the following step is suggested.

Not before 1 hour, and preferably the next day or later, repeat the procedure, with the pressure at 50 mm. of mercury for 15 minutes. Note the number of petechiae. Do not double this number, as in step 1, but add it to the results of step 1.

If the sum of steps 1 and 2 exceed 12, the result is considered abnormal.

Method III, Dalldorf¹⁵

Normal Values: 94% of normal adults show no petechiae in a range of 100 to 200 mm. of mercury.

Apply negative pressure for 1 minute over a skin area 1 cm. in diameter, using a suction cup.

Apply various negative pressures, beginning with less than 100 mm. of mercury, until at least 2 petechiae appear. This end point is considered the level of capillary fragility.

Values below 100 mm. of suction pressure are considered abnormal.

Method IV, Petechiometer Method*

The apparatus consists of a plastic suction cup* 2 cm. in diameter, with a built-in spring-activated suction piston. A simple locking device enables adjustments of negative pressures at 10, 20, and 30 cm. of negative pressure. A built-in magnifying lens enables visualization of the area subjected to the test. The kit also contains a plastic circular disk the size of the suction cup, with the central area marked off by a circle 1 cm. in diameter.

Inspect a nonhairy skin area, either in the subclavicular fossa or the inner aspect of the arm, for blemishes which might be mistaken for petechiae. Note and eliminate from the counts.

Rim the suction cup with lubricant to establish an airtight seal, and then apply it to the skin at 20 mm. negative pressure for 1 minute.

Release the pressure and place the circular disk on the skin area.

Observe only the central 1 cm. area for petechiae. If less than 4 petechiae appear, the capillary resistance is normal. If 4 to 8 petechiae are present, the test is equivocal, and is repeated with 30 cm. negative pressure at another site. More than 8 petechiae

*Supplied by the Rexall Drug Co., 8480 Beverly Blvd., Los Angeles, Calif.

¹⁴Gothlin, G. F.: *Acta paediat.* 20: 71, 1937.

¹⁵Dalldorf, G.: *Am. J. Dis. Child.* 46: 794, 1933.

should appear at this level of pressure for the test to be considered positive. If more than 8 petechiae appear at -20 cm. pressure in the original test, repetition at -10 cm. at another site simply elaborates on how fragile the capillaries are.

We have had considerable experience with the "petechiometer" and have found it simple, inexpensive, and very practical.

Comments on Capillary Fragility.—

Capillary fragility (or capillary resistance) is a reflection of the state of the vascular bed, one of the lines of defense in hemostasis. The vascular tree may be affected by a wide variety of factors which may or may not be related to the clotting mechanism. As a result, abnormalities in capillary fragility must be interpreted as nonspecific phenomena indicating the need for further investigation of the patient.

In the group of "capillary purpuras" with normal platelet counts, the capillary fragility is variable but at times may be markedly abnormal. We have seen mechanical purpura resulting from application of a tourniquet or blood pressure cuff or associated with wearing of tight garters in patients in whom the capillary fragility test has been generally normal. An occasional patient has been found, in this manner, with markedly increased capillary fragility test and no other abnormality. Griffin¹⁶ has emphasized the abnormal Gothlin indices in a large percentage of patients with diabetes and hypertension. He claims reversal of the abnormality by administration of rutin or other flavanoids. Variable values are likewise noted in sepsis, allergy, drug intoxications, scurvy, uremia, senility. Ackroyd¹⁷ maintains that in allergy the basic lesion causing purpura is an increased capillary fragility. Other conditions which may be associated with increased capillary fragility without coexisting abnormality of clotting mechanism include hereditary capillary fragility, hereditary capillary telangiectasia, Glanzmann's thrombasthenia, Kaposi's sarcoma, rheumatic heart disease, and leukemia.

Thrombocytopenia is almost universally associated with an increased capillary fragility. This relationship obtains whether the thrombopenia is primary or is secondary to disease, drug intoxication, etc. In such instances the capillary fragility parallels the level of the platelet count and the skin bleeding time. Exceptions to such parallelism are occasionally found. As a rule, the capillary fragility test reflects the severity of the disease and usually returns to normal in patients whose disease improves with treatment, whether the therapy be splenectomy or the removal of secondary factors. Occasionally the capillary fragility may remain abnormal in patients who appear clinically cured by splenectomy.

In occasional cases of polycythemia vera, even with a high platelet count, increased capillary fragility is seen.

In contrast with thrombocytopenia there are very gross abnormalities of the clotting mechanism which are generally associated with normal capillary fragility. These include hemophilia and hemophilia-like diseases (AHG, PTC, and PTA deficiencies) and fibrinogenopenia.

¹⁶Griffin, J. Q.: *Rutin and Related Flavonoids*, Easton, Pa., 1955, Mack Publishing Co.

¹⁷Ackroyd, J. F.: *Am. J. Med.* 14: 605, 1953.

Hypoprothrombinemia, due to any cause, may be associated with abnormalities of capillary fragility if the prothrombin level sinks low enough. When the prothrombin status is returned to normal by therapy with vitamin K, the capillary fragility follows the same course.

II. Blood Platelet Counting

(A) STAINING BLOOD PLATELETS

Gradwohl Modification of Fonio's Method. Method of Choice

Reagent.—

3.8% Sterilized Solution of Sodium Citrate.—

Dissolve 3.8 gm. of sodium citrate in 100 c.c. of distilled water.

Filter through filter paper, and sterilize in 1 c.c. quantities in ampules, or in larger quantities in bottles. Use 15 pounds pressure in an autoclave for 30 minutes.

Technic.—

Produce hyperemia by immersing the hand in hot water, and then dry thoroughly. Cleanse the finger with alcohol and dry. Puncture in the usual manner and wipe *dry*.

Place a small drop of 3.8% sterilized sodium citrate solution over the puncture, and allow a small drop of blood to ooze through the drop. If desired, the drop of sterile solution may be placed on the disinfected finger and the puncture made through the drop.

There should be about 5 parts of sodium citrate solution and 1 part of blood. Mix the blood and citrate solution on the finger and *scoop* up with the blunt end of the puncturing needle.

Place on the end of a clean, fat-free glass slide and spread with a cover glass in the usual manner for making blood spreads. The film must not be too thin because it will be difficult to stain and to count. If too thick, the erythrocytes are not separated and counting is impossible.

To Stain With Giemsa Stain. Fix for 3 to 5 minutes in pure methyl alcohol. Blow dry, then immerse in a glass of water for 3 to 5 minutes to remove any of the sodium citrate that may remain on the slide.

Place on a staining bridge and cover with dilute Giemsa stain (1 drop to each c.c. of neutral distilled water). Stain for 30 minutes. Wash with neutral distilled water. Examine under the low power of the microscope to ascertain if the slide is stained deeply enough. If it requires further staining, cover with freshly diluted Giemsa stain for another 30-minute period, then wash and allow to dry in the air.

If Wright Stain Is Used. Omit the fixing and washing steps prior to the staining.

There should be no precipitate on the slide, and no clumping of the platelets. If platelets occur in groups the blood came in contact with the air before being mixed with the sodium citrate solution, and the count will be inaccurate. A little practice makes one skillful in making these preparations.

Count as described on page 671.

Method of Dameshek¹⁸

Reagent.—

Dissolve 0.15 gm. of brilliant cresyl blue in 100 c.c. of distilled water containing 8 gm. of sucrose and 0.4 gm. of sodium citrate.

Mix well and filter.

Add 3 drops of a 1:10 solution of Formalin (40%) as a preservative.

Technic.—

Discard the first drop of blood obtained by puncturing the skin.

Place a fairly large drop of the diluting fluid over the puncture wound and gently press the finger so that a small amount of blood wells up into the drop of staining solution.

The proportion of blood to stain should be about 1 to 5.

¹⁸Dameshek, W.: Arch. Int. Med. 50: 579, 1932.

Transfer the mixture of blood and stain to a cover glass, then drop the cover glass on a slide.

Wait 15 to 45 minutes.

Examine with an oil immersion objective.

Count as directed below.

Reticulocytes can be counted in the same preparation.

Method of Fonio

Reagent.—

14% Magnesium Sulphate.—Dissolve 14 grams magnesium sulphate in 50 c.c. distilled water. Dilute to 100 c.c. with distilled water.

Technic.—

Cleanse the patient's finger with ether. Place 1 drop of 14% magnesium sulphate on the tip of the finger. Puncture through the magnesium sulphate. Allow the blood to ooze out and to mix with the magnesium sulphate until a mixture of about 1 part of blood to 9 parts of magnesium sulphate is obtained. Mix by means of the puncture point. Scoop up a drop of the mixture and place on a slide. Draw out with a cover glass in the manner of making a blood film. Allow to dry quickly.

Fix in methyl alcohol for 5 minutes. Dry in the air. Stain for 1 hour with dilute Giemsa stain (2 drops to every c.c.). Wash with neutral distilled water and dry.

Count as directed below.

(B) COUNTING BLOOD PLATELETS

Count all the erythrocytes and blood platelets in consecutive fields until 250 erythrocytes have been counted. Count four such fields after the four-field meander method. (Page 626.)

Calculate the number of blood platelets in 1 cu. mm. of blood by the following formula:

$$\frac{\text{Patient's red count}}{1000} \times \text{No. blood platelets counted} = \text{No. blood platelets in 1 cu. mm. blood.}$$

Example.—

$$\begin{array}{l} 1,000 \text{ red blood cells counted. } 65 \text{ blood platelets counted. Patient's} \\ \text{erythrocyte count } 5,000,000. \\ \frac{5,000,000 \times 65}{1,000} = 325,000 \text{ blood platelets in 1 cu. mm. blood.} \end{array}$$

(C) NORMAL BLOOD PLATELET COUNT VALUES

The normal blood platelet count for the Gradwohl modification of the Fonio method and for the Fonio method is 250,000 to 300,000 platelets per cu. mm. of blood, slightly higher in men than in women and children, or 50 to 65 per 1,000 erythrocytes.

The normal platelet count with the Dameshek method is 500,000 to 900,000, average 716,000 per cu. mm.

Blood platelet counts are made to aid in differentiating the hemorrhagic diseases.

III. Clot Retraction

There are many modifications of the clot retraction test, which at best is only a rough screening test of the clotting mechanism.

Obtain 2 to 3 c.c. of blood by venepuncture and transfer to a clean test tube, preferably a Wassermann tube.

After a solid clot has formed, rim the upper layer with an applicator stick and observe the tube at 1, 2, and 24 hours.

Normally, retraction begins in 1 hour and a definite margin of serum is seen between the clot and the test tube walls in 2 hours. At the end of 24 hours, the clot contracts fully, leaving an area of clear serum above it and surrounding its sides.

Clot retraction is dependent mainly upon two factors: blood platelets and plasma coagulation factors. It was thought at one time that clot retraction depended wholly on the number of platelets. Usually the platelet count is below 100,000 per cu. mm. in most specimens of blood which do not retract, but occasionally poor retraction is seen when the platelet count is normal, such as occurs in thrombasthenia. We have also observed good clot retraction in exceptional cases in which thrombocytopenia was very marked. However, thrombocytopenic states such as the idiopathic type, acquired type, aplastic anemia, and leukemia are the outstanding situations in which clot retraction is poor. In advanced liver diseases with hypoprothrombinemia, clot retraction may be poor. In hemophilia, the coagulation time is markedly delayed, but once a clot has formed, it retracts normally.

IV. Tests for Abnormalities of the Clotting Mechanism

(A) DETERMINATION OF THE CLOTTING TIME

Method I, Capillary Tube Method

Normal Values: 2 to 4 minutes.

Materials Required.—

3 to 4 inch capillary tubes.

Capillary blood specimen.

Technic.—

Obtain a drop of blood by skin puncture and allow to enter a capillary tube.

Break off small pieces of the tube at 30-second intervals until a fine thread of fibrin persists at the site of separation of fragments of the capillary tube.

The time from skin puncture to the formation of the fibrin thread is the clotting time.

Method II, Modified Lee-White Method

Normal Values: 5 to 20 minutes.

Materials Required.—

3 clean test tubes rinsed with 0.85% saline.

Sterile clean syringe and needle rinsed with sterile 0.85% saline.

Technic.—

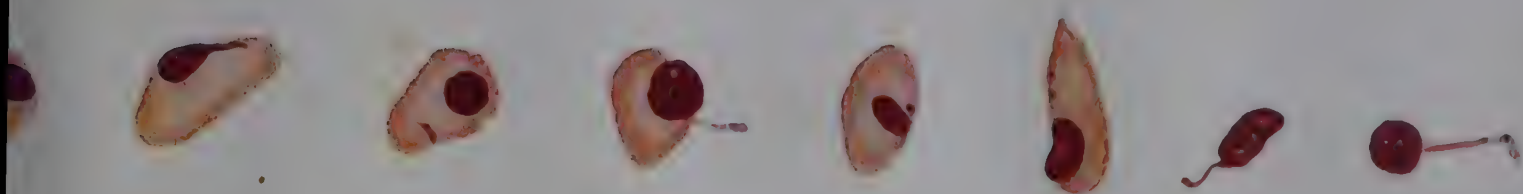
Draw blood from a vein by a clean puncture, avoiding an undue amount of suction.

Remove the needle from the syringe and gently transfer 1 c.c. quantities of the blood specimen to each of the 3 test tubes.

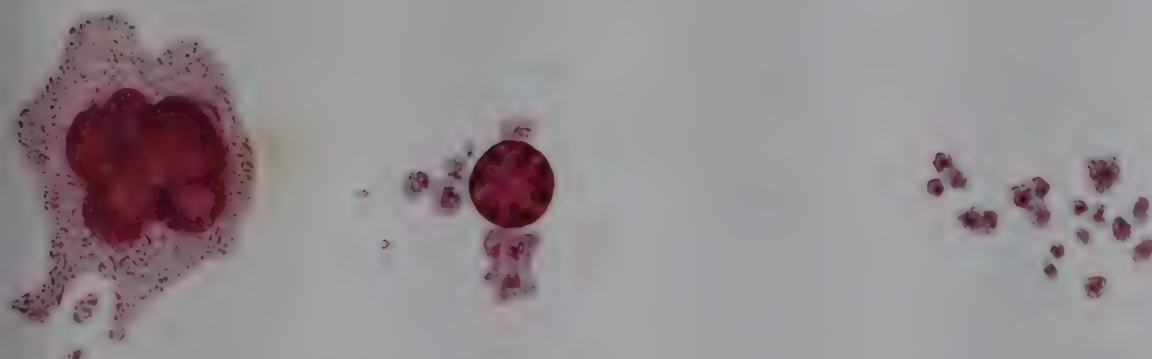
After approximately 5 minutes, gently tilt one of the tubes and observe for clotting. If a clot has not formed, tilt this same tube every minute until the blood is no longer liquid. At about 1 minute later, check the second tube for clot formation. If this tube shows a firm clot, check the third tube. If it does not clot, tilt this second tube every 30 seconds until a firm clot is detected, and then check the third tube.

The clotting time is that time which has elapsed between the venepuncture and a fully developed clot in the 3 tubes.

THEORIES OF BLOOD PLATELET DEVELOPMENT

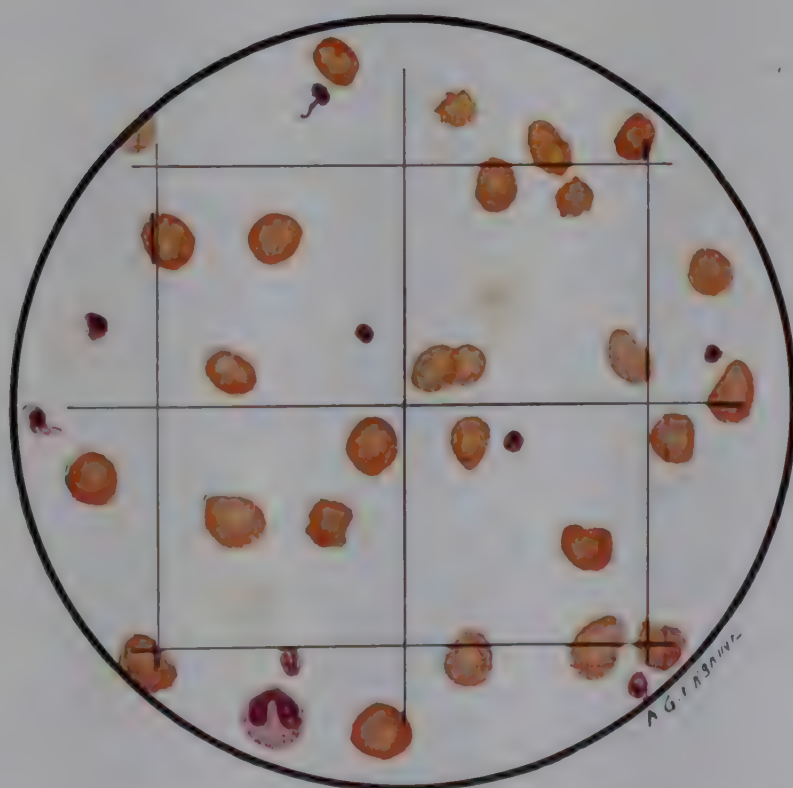


HILLING — EXTRUSION OF NUCLEUS FROM A NUCLEATED ERYTHROCYTE



RIGHT — DEVELOPMENT FROM A MEGAKARYOCYTE

BLOOD PLATELET COUNTING



GRADWOHL MODIFICATION OF THE METHOD OF FONIO

Method III

Various modifications of tube methods for determining the clotting time have been devised, and each has its champion. These modifications have been outlined by Albritton¹ as follows:

METHOD	TEMPERATURE	CLOTTING TIME RANGE IN MINUTES
1 c.c. blood in each of four tubes	Room	5 to 20
1 c.c. blood in paraffined tube	37° C.	16 to 43
1 c.c. blood in siliconed tube	Room	22 to 61
1 c.c. blood in Lusteroid tubes	37° C.	25 to 50

Method IV, Plasma Recalcification Method,² Quick**Normal Values:**

Slow centrifugation: 95 to 135 seconds.

Fast centrifugation: Not more than 15 seconds longer than in slow centrifugation.

Materials Required.—

M/10 Sodium Oxalate.—Prepared by dissolving 1.34 gm. of sodium oxalate in 100 c.c. of distilled water (total).

M/100 Calcium Chloride.—Prepared by dissolving 0.11 gm. of anhydrous calcium chloride and 0.42 gm. sodium chloride in 100 c.c. of distilled water.

Centrifuge, stop watch, and venous blood.

Technic.—

Draw 4.5 c.c. of blood by clean venepuncture, remove needle from syringe, and immediately transfer blood to 0.5 c.c. of oxalate solution.

Mix thoroughly by inverting stoppered tube several times.

Divide the mixture into two approximately equal portions.

Centrifuge one portion at 1,000 r.p.m. for 5 minutes (platelet-rich plasma), and the other at 3,000 r.p.m. for 5 minutes (platelet-poor plasma).

Withdraw 0.1 c.c. of plasma from each specimen and place in separate test tubes in a water bath at 37° C.

Add 0.2 c.c. of M/100 calcium chloride to each sample of plasma and begin timing immediately with a stop watch.

Keep the tubes in a water bath and tilt occasionally until a clot forms. This is the end point of the test. Note the exact time of clot formation.

The recalcified plasma clotting time is prolonged in marked hypoprothrombinemia. In deficiencies of antihemophilic globulin (AHG), plasma thromboplastin component (PTC), and plasma thromboplastin antecedent (PTA), the clotting time of recalcified plasma is also prolonged. The degree of prolongation appears to be related to the degree of deficiency. A prominent feature of these deficiencies is that the clotting time of the platelet-poor (rapidly centrifuged) plasma shows a much greater prolongation of clotting time than that of the platelet-rich plasma.

Method V, Heparin-Retarded Blood Coagulation Time of de Takats³

Normal Values: 20 to 35 minutes; average 27.5 minutes.

Materials Required.—

Heparin solution containing 0.004 mg. per 0.1 c.c. of isotonic saline. This can be made by diluting the standard 10 mg. per c.c. heparin solution 1:250 with isotonic saline.

¹Albritton, E. C.: *Standard Values in Blood*, Philadelphia, 1952, W. B. Saunders Co., p. 16.

²Quick, A. J.: *Am. J. M. Sc.* 201: 469, 1941.

³de Takats, G.: *Angiology* 1: 317, 1950; *Surg., Gynec. & Obst.* 77: 31, 1943.

Venous blood freshly drawn in a dry syringe. Discard the first and last c.c. of blood to prevent admixture with tissue juices.

Technic.—

Place 0.1 c.c. of heparin solution in a clean dry glass test tube in a water bath at 37° C.

Add 1 c.c. of blood to this tube and invert twice.

At the end of 12 minutes, tilt the tube gently at 1-minute intervals until a firm clot has formed. The end point occurs when the tube can be completely inverted without any fluid breaking away from the clot.

Rosenthal states that the "heparin clotting time appears to be the most valuable single test or index of coagulation." He emphasizes that the test may be used in conditions associated with rapid clotting processes as well as those having prolongation of coagulation. Prolonged heparin clotting times were found in the deficiency states involving AHG, PTC, and PTA. In these conditions the heparin clotting test was found more sensitive than standard procedures such as the Lee-White technic. In addition to these classical conditions, Rosenthal found that 50 per cent of patients with chronic myelocytic or lymphocytic leukemia had prolongation of the heparin clotting time. This previously unreported coagulation defect was closely related to the degree of hemorrhagic diathesis in these patients even though they did not have thrombocytopenia of a degree usually considered critical.

The heparin clotting time was found prolonged in some patients with bone marrow depression and slight thrombocytopenia, such as may occur in malignant lymphomata and other diseases related to the hematopoietic system. Although these patients showed no clinical manifestations of hemorrhage, they had prolongation of the heparin clotting time.

Rosenthal^{1, 2} found evidence of accelerated coagulability in 74 per cent of patients within three weeks following the onset of myocardial infarction. The heparin clotting time was shortened in these patients with a mean of 18.7 minutes, ranging from 11 to 20 minutes. Routine clotting times and platelet counts were normal. A control group of patients with cardiovascular disease but without myocardial infarction showed normal values. Dicumarol was found to have marked effect in prolonging the heparin clotting time.

Method VI, Calcium Time of Blood

Have on hand tubes with a diameter of 8 to 10 mm.

Remove about 2 or 3 c.c. of blood from the vein in the usual manner. Place 1 c.c. of blood in each of 2 small tubes.

To one of the tubes, add 3 drops of 1% solution of calcium chloride.

Add nothing to the other tube.

Observe the time required for coagulation to take place in both tubes. If coagulation takes place in the tube containing the calcium chloride before coagulation in the tube without the calcium, then the patient's delayed coagulation time is due to a deficiency of calcium. If coagulation in both tubes takes place within the normal range of time, calcium is not deficient.

Comments on Clotting Time Determinations.—

Measurement of the clotting time may or may not disclose the presence of an abnormality in the clotting mechanism. Finding a prolonged clotting time

¹Rosenthal, R. L.: Bull. New York Acad. Med. 28: 607, 1952.

²Rosenthal, R. L., and Weaver, J. C.: Circulation 6: 257, 1952.

should always initiate further investigation of disturbances in coagulation. Since prolongation of the clotting time may be due to deficiencies of plasma factors, such as AHG, PTC, PTA, fibrinogen, accelerator factors, and prothrombin, special procedures directed at determining these specific defects must be pursued. Anticoagulants, either acquired or idiopathic, may have to be considered. A screening procedure for determining whether dealing with a deficiency or an anticoagulant has been described by several authors,¹⁻⁷ using technics of matching mixtures of blood.

(B) DIFFERENTIATION OF PLASMA DEFECTS AND CIRCULATING ANTICOAGULANTS

Principle.—When an abnormal clotting time has been found, the question arises as to whether it is due to a plasma defect or to a circulating anticoagulant. Differentiation of these two mechanisms can be made by determining the coagulation time of mixtures of normal and abnormal blood.

Materials Required.—

These are the same as in the plasma recalcification method of Quick, page 673.

Technic (Adapted to the Method of Cartwrights).—

Add 4.5 c.c. of venous blood from the patient to 0.5 c.c. of M/10 sodium oxalate.

Repeat this procedure in another tube, using blood of a normal subject.

Mix each sample thoroughly.

Centrifuge both specimens of oxalated blood in the same centrifuge at 1,000 r.p.m. for 5 minutes.

Prepare a series of mixtures of the two plasmas as follows:

TUBE NUMBER	1	2	3	4	5	6
Patient plasma (c.c.)	0.2	0.4	0.1	0.1	0.1	0.0
Normal plasma (c.c.)	0.0	0.1	0.1	0.2	0.4	0.2

Mix the contents of each tube thoroughly.

Transfer 0.1 c.c. samples of each tube to 6 clean test tubes and place in a water bath at 37° C.

Add 0.2 c.c. of the M/100 calcium chloride to each tube, mix, and begin timing immediately with a stop watch.

Observe each tube for firm clot formation and record time.

Tube 6 is the **normal control** and should have a clotting time of 95 to 135 seconds.

Tube 1 should have a clotting time prolonged beyond 135 seconds.

If the patient's condition is due to a deficiency in some plasma factor necessary for coagulation, the addition of oxalated normal plasma will correct the deficiency, and under such conditions tubes 2, 3, 4, and 5 will have normal coagulation times.

If the disorder in the patient is due to a circulating anticoagulant, tubes 2, 3, 4, and 5 will show increased coagulation times with progressively diminishing values from tubes 2 to 5.

¹Graham, J. B., Buckwalter, J. A., Hartley, L. J., and Brinkhous, K. M.: *J. Exper. Med.* 90: 97, 1949.

²Merskey, C.: *J. Clin. Path.* 3: 301, 1950.

³Stefanini, M., and Crosby, W. H.: *Proc. Soc. Exper. Biol. & Med.* 73: 301, 1950.

⁴Susman, L. N., Wald, N., and Rosenthal, R. L.: *Blood* 1: 1100, 1952.

⁵Ham, T. H.: *A Syllabus of Laboratory Examinations*, Cambridge, Mass., 1950, Harvard University Press.

⁶Rosenthal, R. L.: *Am. J. Med.* 17: 57, 1954.

⁷Brinkhous, K. M., Langdell, R. D., Penick, G. D., Graham, J. B., and Wagner, R. H.: *J. A. M. A.* 154: 481, 1954.

⁸Cartwright, G. E.: *Diagnostic Laboratory Hematology*, New York, 1954, Grune & Stratton, p. 64.

After ascertaining whether the coagulation defect is due to deficiency of plasma factors or to circulating anticoagulant, special tests must be carried out to ascertain the specific elements involved. These include the following procedures:

A. Tests for Plasma Deficiencies:

1. Partial thromboplastin time (PTT)¹
2. Assay of antihemophilic factor¹
3. Prothrombin time
4. Prothrombin consumption time (plasma or serum)
5. Studies of mixtures
6. Fibrinogen determination
7. Thromboplastinogen activity test²
8. Thromboplastin generation test³

B. Tests for Anticoagulants:

1. Antithrombin and antithromboplastin⁴
2. Hyperheparinemia⁵

(C) TESTS FOR PLASMA DEFICIENCIES

PROTHROMBIN TIME DETERMINATIONS

Method I, Method of Quick⁶

Reagents.—

M/10 Sodium Oxalate.—

Dissolve 1.34 gm. of sodium oxalate in enough distilled water to make a total of 100 c.c.

M/40 Calcium Chloride.—

Dissolve 0.28 gm. of anhydrous calcium chloride in enough distilled water to make a total of 100 c.c.

Thromboplastin Solution.—

Use the brain of a freshly killed rabbit. Free it of the large superficial blood vessels, wash, then grind to a paste and spread in a thin layer on a plate glass or a flat dish. Dry thoroughly at 37° C., remove the material from the plate, and preserve in a stoppered container. Mix 0.2 gm. of this material with 0.3 c.c. of 0.85% sodium chloride and incubate at 56° C. 15 minutes, obtaining an emulsion which has practically maximum activity; i.e., it will, when added to human plasma, cause clotting in 11 to 12½ seconds. Any preparation which fails to show this activity is discarded.

Technic.—

Place 1 c.c. of M/10 sodium oxalate in a test tube.

Withdraw blood from a vein rapidly and with special precaution to avoid trauma, add 9 c.c. of blood to the sodium oxalate, and mix to prevent clotting.

Centrifuge at low speed for 5 minutes. Withdraw plasma with a clean dropper, placing in a clean, dry test tube.

Transfer 0.1 c.c. of this plasma to a dry clean Kahn tube and mix with 0.1 c.c. of thromboplastin solution.

Add 0.1 c.c. of M/40 calcium chloride, shake quickly, and place in a water bath kept at 37° C. Record the exact time required for the formation of a solid clot.

¹Langdell, R. D., Wagner, R. H., and Brinkhous, K. M.: *J. Lab. & Clin. Med.* **41**: 637, 1953.

²Quick, A. J., Hussey, C. V., and Stapp, W. F.: *J. Lab. & Clin. Med.* **30**: 142, 1952.

³Biggs, R.: *J. Clin. Path.* **6**: 23, 1953.

⁴Quick, A. J.: *The Hemorrhagic Diseases and Physiology of Hemostasis*, Baltimore, 1942, Charles C Thomas.

⁵Allen, J. G., Bogardus, G., Jacobson, L. O., and Spurr, C. L.: *Ann. Int. Med.* **27**: 382, 1947.

⁶Quick, A. J.: *Am. J. Clin. Path.* **10**: 222, 1940.

With an active preparation of thromboplastin, normal human plasma will clot consistently in 11 to 12½ seconds.

Comments on the Quick One-Stage Method of Prothrombin Time.—

Since Quick's original publication, numerous modifications of the method have been devised in order to simplify the procedure for routine clinical laboratory use, improve on the reagents, or correct certain objections to the basic principles in the test.

In general, very few laboratories prepare their own thromboplastin extracts since satisfactory preparations are now commercially available. The activity of a given batch of thromboplastin should be checked against normal controls. To circumvent the possibility that the so-called "normals" are not truly normal, commercial preparations of lyophilized plasma are available as controls.¹ The prothrombin time of an unknown sample is greatly dependent on the thromboplastin potency and should be reported in seconds, with an accompanying note of the time for the normal control.

Curves are constructed with varying dilutions of plasma, with time (seconds) as one component and percentages as another. Such calibration curves are used as references for reporting tests in percentages. An objection to the method as originally reported by Quick is that, in the higher percentage ranges, the slope of the curve is so steep that small increments of time reflect large changes in percentages. To circumvent this objection, various dilutions of the test plasma have been suggested. Usually the dilution is in the range of 1:10 but may be adjusted at other levels for special situations. Although saline has been recommended as the diluent by some, it is now generally agreed that it should not be used because it dilutes all coagulation factors in plasma. Prothrombin-free plasma is one of the diluents most universally recommended. There is some question as to whether this should be human or animal plasma. The adsorbents used for preparation of such prothrombin-free plasma have been barium sulphate or passage through filter paper pads containing high concentrations of asbestos.

A method which considers many of these factors is that of Rosenfield and Tuft² as modified by Alexander and deVries³ and is recommended.

Method II, Prothrombin Time, Rosenfield and Tuft,² Modified by Alexander and deVries³

Reagents.—

M/10 Sodium Oxalate.—

Dissolve 1.34 gm. anhydrous sodium oxalate
in 100 c.c. distilled water.

M/10 Potassium Oxalate.—

Dissolve 8.423 gm. crystalline potassium oxalate in distilled water and dilute to 1,000 c.c. with distilled water.

Use 1 c.c. of this solution as anticoagulant for 9 c.c. of blood. This is used for the preparation of prothrombin-free plasma.

M/40 Calcium Chloride.—

¹Hades, M. D.: Clin. Chem. 5: 59, 1953. (Preparation was the Diagnostic Plasma, Warner-Chilcott Laboratories.)

²Rosenfield, R. E., and Tuft, H. S.: Am. J. Clin. Path. 17: 405, 1947.

³Alexander, B., and deVries, A.: J. Clin. Invest. 28: 24, 1949.

Dissolve 2.77 gm. purified anhydrous calcium chloride in distilled water and dilute to 1,000 c.c. with distilled water.

Thromboplastin.—See Quick method above, and comments.

Prothrombin-Free Human Plasma.—

Collect 9 c.c. of blood from each of at least 5 normal subjects and place in 1 c.c. of M/10 potassium oxalate and mix.

Separate the plasma and pool.

Divide the plasma pool into 2 portions, using one for adsorption with barium sulphate and the other for diluting the normal or test plasma.

Add 100 mg. of barium sulphate powder for each c.c. of plasma, shake thoroughly, and incubate for 10 minutes at 37° C.

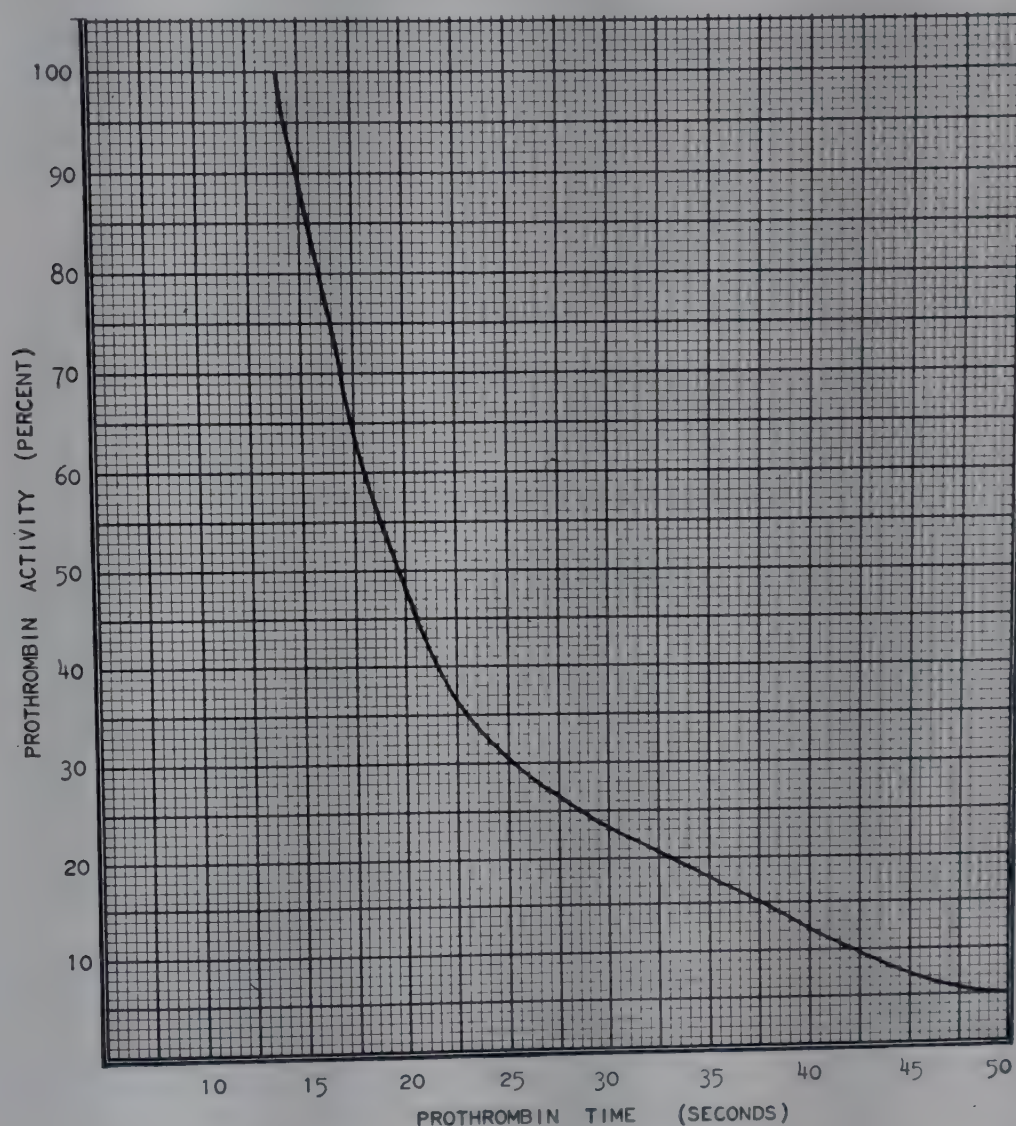


Fig. 176.—Curve constructed for the Quick prothrombin time determination. Such calibration curves are used as references in reporting prothrombin time in percentage.

Centrifuge at 3,000 r.p.m. for 30 minutes, and pour off the supernatant fluid.

Refrigerate this at 4° C. until ready for use. Since this material must be used within 2 hours after its preparation, Frommeyer⁴ recommends lyophilization in order to maintain its potency.

Lyophilized prothrombin-free plasma is reconstituted with buffer at the time of its use.

Preparation of Curve for Time/Percentage Relationships.—Dilute *fresh, pooled, normal* plasma serially with the prothrombin-free plasma prepared as above. On each dilution perform a Quick prothrombin time test (page 676) and plot a curve with the time in seconds against the concentration in per cent. (Fig. 176.)

⁴Frommeyer, W. B.: *J. Lab. & Clin. Med.* 24: 1356, 1949.

Technic.—

Place 0.5 c.c. of M/10 sodium citrate in a test tube.

Add 4.5 c.c. of venous blood and mix.

Centrifuge at 1,000 r.p.m. for 5 minutes.

Remove the supernatant plasma with a clean pipette and transfer it to a clean test tube.

Place all the reagents and tubes used hereafter in a water bath at 37° C.

Place 0.1 c.c. of the plasma and thromboplastin solution in a clean Kahn tube and shake gently to insure mixing.

With stop watch in hand, blow in 0.1 c.c. of the M/40 calcium chloride solution, and begin timing.

Remove the tube from the water bath periodically, and observe the time required for a clot to form; check the concentration in percentage on the curve prepared as above.

Comments: The first steps in prothrombin time determination with this test are essentially the Quick technic, with a time-concentration curve prepared by dilution with prothrombin-free plasma. If the result of this determination falls in the flat part of the time-concentration curve, 25 seconds or longer, the result may be recorded as a final figure. However, if a shorter time is consumed, it is recommended that the unknown plasma sample be diluted 1:10 with the prothrombin-free plasma, *NOT SALINE*, and the prothrombin time on a 0.1 c.c. aliquot of this be determined as in the method for undiluted plasma. The result in per cent concentration is then multiplied by 10.

Method III, Prothrombin Time, Shapiro et al., Using Whole and Diluted Plasma^{1, 2}

The estimation of prothrombin levels is reliable only if established conditions are maintained constantly and manipulative characteristics are adhered to most rigidly.

One of the major objections to single-stage technics is that large increments of prothrombin as expressed in percentage are covered by short differences in time. This is particularly true in the range of 60 to 30 per cent. To obviate this difficulty, dilution of plasma has been recommended by a number of workers. This Link-Shapiro modification takes this into account. Shapiro uses saline as a diluent for plasma, whereas others (see the preceding method) use other diluents, such as prothrombin-free plasma.

The difference between this method and the single-stage technic is that the method of Shapiro includes estimation of both whole and diluted (12.5%) plasma prothrombin time.

Apparatus.—

Fig. 177 illustrates the type of apparatus needed for the procedure. It permits all reagents and plasma to be maintained at a constant temperature throughout the entire period of the test. The glass wall of the tank enables the operator to observe the changes visible in the test tube. A precise and unmistakable end point is obtained by lifting the clot with a tiny hook the instant it forms from the otherwise clear fluid.

The stop watch is operated by a foot treadle, leaving the hands free to manipulate the test.

¹Shapiro, S., Sherwin, B., Redish, M., and Campbell, H. A.: *Proc. Soc. Exper. Biol. & Med.* 50: 85, 1942.

²Shapiro, Shepard: *Exper. Med. & Surg.* 2: 103, 1944.

Reagents.—**M/10 Sodium Oxalate.—**

Dissolve 13.4 gm. of sodium oxalate, reagent grade, in distilled water in a liter volumetric flask and dilute to 1,000 c.c. with distilled water.

0.85% Sodium Chloride.—

Dissolve 8.5 gm. of sodium chloride, reagent grade, in enough distilled water to make 1,000 c.c. of solution.

0.025 M Calcium Chloride.—

Dissolve 2.77 gm. of calcium chloride, reagent grade, in distilled water in a liter volumetric flask and dilute to 1,000 c.c. with distilled water.

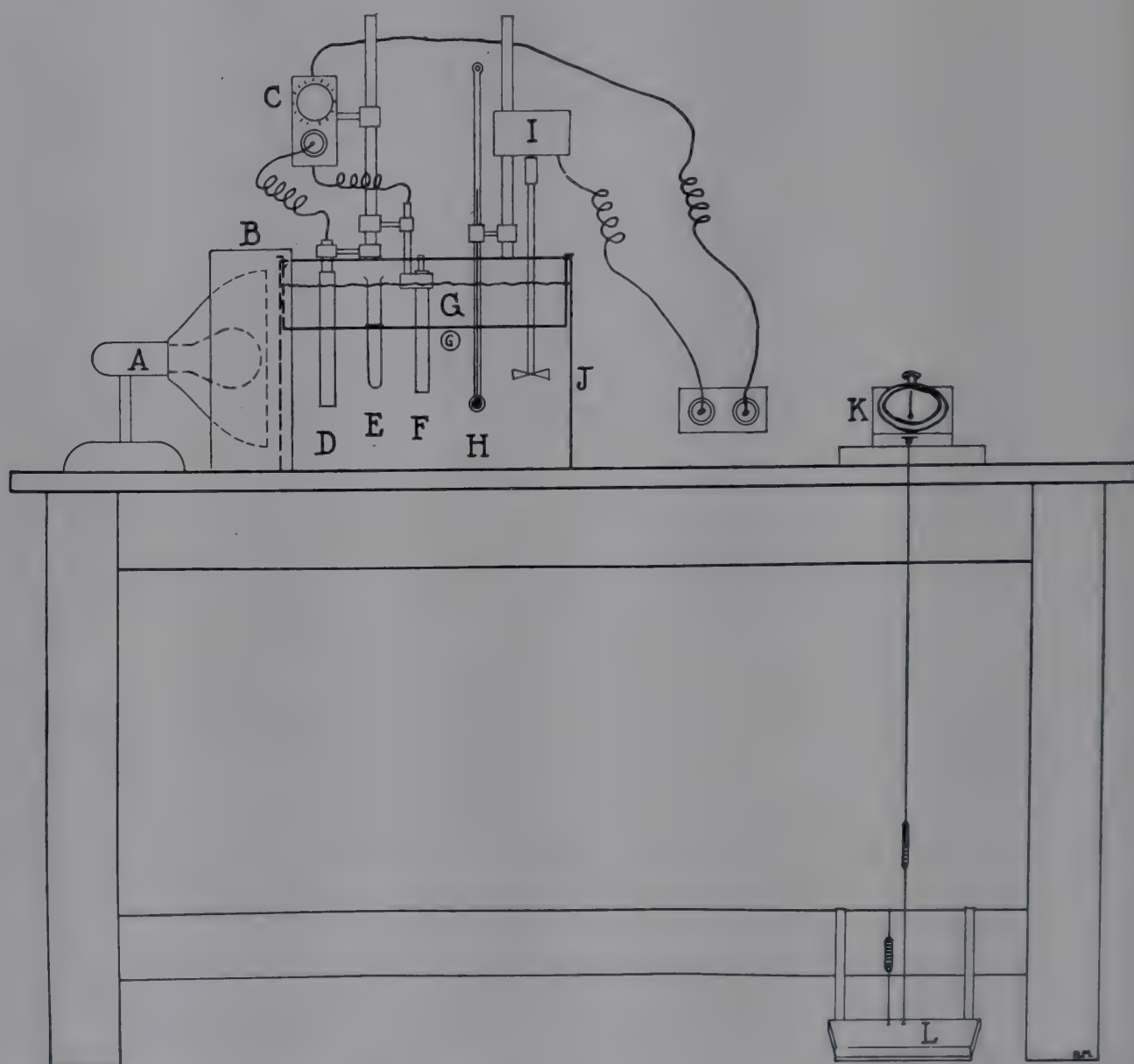


Fig. 177.—Shapiro apparatus for prothrombin time using whole and diluted plasma. A, 60-watt lamp; B, reflector; C, electric bulb indicator; D, immersion heater; E, test tube suspended on perforated rack G; F, thermostat set at 37.5° C.; G, perforated rack; H, low range thermometer; I, electric stirrer; J, all glass tank; K, stop watch; L, pedal.

Thromboplastin.—

Sacrifice a rabbit by air embolism. Quickly remove the lungs and desiccate in vacuo. Pulverize the dried tissue and store in a refrigerator.

Place 50 mg. of this thromboplastin powder in a small centrifuge tube and add 2.5 c.c. of 0.85% sodium chloride.

Stir until a homogeneous suspension is obtained. Stir the mixture constantly while maintaining it at 54° to 56° C. in a water bath for 10 minutes. Cool to 25° to 26° C.

Add 2.5 c.c. of 0.025 M calcium chloride solution. Stir for 4 minutes. Centrifuge. Stop the centrifuge gradually to avoid resuspending the flocculent precipitate. Remove the slightly turbid supernatant fluid with a capillary pipette. Use this in the determination.

Simplastin* is the thromboplastin of choice. It requires only the addition of distilled water. It contains the calcium chloride.

Technic.—

With sterile precautions place 0.5 c.c. of M/10 sodium oxalate in a 5 c.c. syringe. Withdraw 4.5 c.c. of venous (or arterial) blood and mix instantly.

Centrifuge at 1,700 r.p.m. for 10 minutes.

Transfer the clear plasma to another clean dry test tube properly labeled. Set aside a portion of the plasma in a test tube 75 by 10 mm.

Place 0.1 c.c. of plasma in another 75 by 10 mm. tube.

Add 0.7 c.c. of 0.85% sodium chloride solution. Mix the diluted (12.5%) plasma thoroughly.

In order to mix the plasma thoroughly without contamination, hold the test tube firmly near the top with the thumb and index finger while striking the bottom of the tube gently with the index finger of the other hand. Place both tubes (diluted and undiluted plasma) in a water bath at a constant temperature of 37° to 38° C.

Use Hagedorn-Jensen microblood sugar pipettes in the manipulation.

Transfer 0.2 c.c. of thromboplastin or thromboplastin-calcium chloride suspension to test tubes 100 by 12 mm. Use a 0.2 c.c. pipette. Suspend the tubes in a rack in the constant temperature bath for 10 minutes.

Shake the plasma again for an instant and quickly add 0.1 c.c. of whole plasma to the 0.2 c.c. of thromboplastin or thromboplastin-calcium chloride mixture. Start the stop watch as the plasma is quickly blown from the pipette. Tap the tube sharply to mix the solutions and to insure uniform initiation of the clotting process. Using a small stirrer made of No. 22 nichrome wire with a tiny triangular-shaped loop at the end, test the mixture every half second. Pass the stirrer rhythmically from side to side of the tube so that it is swept through the liquid mixture every half second. The end point is the formation of a fibrin clot. The clot adheres to the stirrer and becomes separated from the fluid as it is drawn to the wall of the test tube. Stop the stop watch the instant the clot forms. This is the prothrombin clotting time.

The clot is usually somewhat turbid since the calcium oxalate formed upon recalcifying the plasma is enmeshed in the clot. Frequently the solution acquires a viscous appearance due to the formation of fibrils before the true clot forms. This should be disregarded.

Repeat the procedure using 0.1 c.c. of the diluted plasma in place of whole plasma.

Normal.—Whole plasma prothrombin time—15.5 seconds ± 1.5 ; diluted plasma prothrombin time—39.5 seconds ± 2.5 .

The relative sensitivity of the estimation of whole as compared with diluted plasma prothrombin time may be demonstrated by using slightly prothrombinopenic blood induced by salicylates. The prolongation in the whole plasma is not detectable, while in the dilute (12.5%) plasma it is significantly extended, being eight seconds greater than in the whole plasma.

A reduction in the diluted plasma prothrombin time to a level below the normal limits signifies hyperprothrombinemia. The whole plasma prothrombin time rarely falls below the normal limits and consequently does not reveal increases beyond normal of the concentration or activity of prothrombin.

Shapiro has observed in a number of instances of reactive hyperprothrombinemia that the diluted plasma prothrombin time is below normal limits, while the whole plasma prothrombin time is prolonged in excess of normal. In-

*Manufactured by Warner-Chilcott, New York.

creased anticoagulant activity has been observed during the recovery phase of acute thrombotic and thromboembolic processes. It seems to occur frequently at such times and has been found to be a prognostic sign: *Recurrence of thrombosis has not been observed in any of Shapiro's cases in which this phenomenon has been detected.*

Concomitantly with the development of acute thrombosis, especially in venous channels, there occurs a measurable acceleration of the coagulability of the blood as indicated by an elevation in the level or activity of prothrombin. Usually this exceeds normal, reaching a hyperprothrombinemic value. It has been observed also but with less regularity in acute arterial thrombosis such as, for example, in peripheral arterial embolization or in acute coronary thrombosis.

Prolongation of prothrombin time has been noted in some cases, especially when shock phenomena are present. In instances in which serial estimations of prothrombin time have revealed a progressive increase to hyperprothrombinemic levels, recurrence, or extension, of the arterial thrombotic process has been observed.

Method IV, Two-Stage Method of Prothrombin Time, Warner, Brinkhous, and Smith¹

The basic principle of this method is the measurement of thrombin yield against a standardized solution of fibrinogen. Just as with other prothrombin time methods, the two-stage technic has undergone numerous modifications. Its complexity has been a bar to routine clinical use, but it has served as a useful investigative tool.

Method V, P and P Method of Owren and Aas²

Reagents.—

Thromboplastin Derived From Human Brain.—

Free human brain from pia and blood vessels.

Wash, and grind the gray substance in a mortar with 0.9% sodium chloride solution to a fine emulsion. Emulsification is facilitated by applying a mechanical blender.

Dilute the emulsion with 0.9% saline to which 20% Veronal buffer is added. Approximately 5 c.c. solution should be used for each gram of brain substance.

Allow the extract to stand for about 2 hours at 45° C.

Strongly centrifuge for 15 minutes.

Discard the precipitate.

Freeze the grayish colloidal suspension at -20° C. in portions corresponding to the daily requirement. Before using, thaw it at 37° C.

This extract gives a Quick prothrombin time in normal plasma of approximately 15 seconds. When stored frozen at a temperature below -20° C., the extract remains unchanged during several months.

Note: There are a number of good thromboplastin preparations on the market, which can be substituted for this human brain thromboplastin.

Veronal Buffer.—

To 570 c.c. of 0.1 M sodium diethylbarbiturate

add 430 c.c. N/10 hydrochloric acid

and 5.67 gm. sodium chloride.

Dilute with an equal volume of 0.9% sodium chloride solution.

¹(a) Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.* **114**: 667, 1936.

(b) *Idem*: *J. Exper. Med.* **66**: 401, 1937.

(c) *Idem*: *Am. J. Physiol.* **125**: 296, 1939.

²Owren, P. A., and Aas, K.: *Scandinav. J. Clin. & Lab. Invest.* **3**: 201, 1951.

Diluting Solution.—

To 800 c.c. of 0.9% sodium chloride solution
add 200 c.c. of Veronal buffer pH 7.35
and 700 mg. of potassium oxalate monohydrate.³

Calcium Chloride Solution.—

This is usually 0.03 M, but optimal concentration must be determined for each batch of ox plasma.

Prothrombin-Free and Proconvertin-Free Ox Plasma.—

Mix 9 volumes of ox blood with 1 volume of 2.5% (w/v) potassium oxalate monohydrate solution, and obtain plasma by centrifugation.

Remove prothrombin by using asbestos-paper filter pads containing 30% asbestos. Store plasma overnight at 0° C.

Centrifuge to remove fatty material which tends to clog filters.

Pass the plasma through a clarifying filter containing 20% asbestos, and then once through filter paper pads containing 50% asbestos. 400 c.c. of plasma may be passed through a filter of 15 cm. diameter. When larger amounts are filtered, some prothrombin remains in the filtrate.

Adjust the reaction of the plasma to pH 7.3 by adding N/2 hydrochloric acid.

Store at -20° C. or lower temperatures, in small bottles or glass tubes stoppered with corks, in quantities suitable for 1 day's use.

2% Potassium Oxalate Solution.—

Dissolve 2 gm. potassium oxalate in distilled water and dilute to 100 c.c. with distilled water.

Technic.—

Place 0.5 c.c. of 2% potassium oxalate in a test tube.

Add 4.5 c.c. of blood obtained by venepuncture and mix.

Centrifuge for 5 minutes at 1,000 r.p.m. and remove the plasma.

To 0.2 c.c. of plasma add 1.8 c.c. of diluting solution.

Place all the reagents in a water bath at 37° C.

Place 0.2 c.c. of the prothrombin-free, convertin-free ox plasma in a small Kahn tube.

Add 0.2 c.c. of the plasma dilution

and 0.2 c.c. of thromboplastin.

Mix by shaking gently.

With a stop watch ready, blow out 0.2 c.c. of calcium chloride solution and begin timing.

Observe the end point for clotting.

Record the time as in the methods above. Obtain the percentage from a correlation graph as in other methods. Prepare the graph by serial dilutions of normal plasma, using diluting fluid, but maintaining a concentration of 100 mg. % oxalate³

Comment: Owren believes that by following his procedure he maintains the following principles:

1. Determines combined effect of prothrombin and proconvertin.
2. Keeps a constant substrate of thromboplastin, calcium, proaccelerin, and fibrinogen. The latter two come from the added adsorbed ox plasma.

He particularly points out that his "P and P" method is superior to previous methods in the control of Dicumarol therapy, in which the effect is a depression of the prothrombin and proconvertin values, leaving proaccelerin unaltered. In vitamin K deficiency and in liver disease, the same relationships hold, although in the latter, severe damage may cause a decrease in proaccelerin as well.

³Owren, P. A.: *Scandinav. J. Clin. & Lab. Invest.* 1: 81, 1949.

Method VI, The Specific Determination of Prothrombin, Owren¹

The basic principle in this test is keeping the reaction mixture constant in the content of thromboplastin, calcium, proconvertin, proaccelerin, and fibrinogen.

Reagents.—

Reagents are the same as in the "P and P" method (above), with the addition of proconvertin solution.

Proconvertin.—

Proconvertin is obtained from human serum by adding a small amount (3 c.c. per 100 c.c. of blood) of thromboplastin to venous blood, stirring immediately after withdrawal. This results in disappearance of prothrombin within 10 to 30 minutes. Storage at room temperature for 2 days causes accelerin to disappear.

Plasma-Serum Reagent.—

Mix the serum prepared as above with equal parts of prothrombin-free and proconvertin-free ox plasma to make a plasma-serum reagent.

Technic.—

The method is essentially that of the "P and P" method, with the following quantities used in this test:

- 0.2 c.c. of a 1:10 dilution of test plasma.
- 0.4 c.c. of plasma-serum reagent.
- 0.2 c.c. of thromboplastin.
- 0.2 c.c. of calcium chloride.

The time required for clotting in this mixture is recorded. The percentage prothrombin is calculated by reference to a correlation graph prepared in a manner similar to that in the "P and P" method.

Comments: The major point about this modification is that it actually measures the activity of *prothrombin only*, whereas the usual one-stage modified Quick technics and the "P and P" method of Owren measure both prothrombin and proconvertin. In following patients with Dicumarol therapy, the "P and P" method is desirable because both prothrombin and proconvertin are affected. The determination of prothrombin only is a helpful tool in research.

Method VII, The Specific Determination of Proconvertin, Owren

The basic principle involved in this test is keeping the reaction mixture constant in thromboplastin, calcium, proaccelerin, prothrombin, and fibrinogen.

Reagents.—

The thromboplastin and calcium solutions are the same as in the "P and P" test, page 682.

Proconvertin-free ox plasma is prepared by filtering oxalated ox plasma through an asbestos filter pad containing only 20% asbestos.

Technic.—

The procedure is essentially that of the "P and P" method with the following quantities being used:

- 0.2 c.c. proconvertin-free ox plasma.
- 0.2 c.c. thromboplastin.
- 0.2 c.c. unknown plasma diluted 1:20.
- 0.2 c.c. calcium chloride.

The time required for clotting is recorded. The percentage proconvertin is calculated by reference to correlation graphs prepared from normal plasma diluted 1:20.

¹Loc. cit., p. 683.

Comment: This determination has been a helpful tool in the investigation of clotting mechanisms. In clinical studies of bleeding diatheses, Alexander and associates¹ and Owren and Aas² have described congenital hypoproconvertinemia. In 1953, Owren³ described this defect as a congenital and hereditary defect. He points out that the whole clotting time in these patients may be normal or only slightly prolonged. The Quick "prothrombin time" was markedly prolonged but vitamin K did not restore the hypoproconvertinemia to normal.

Method VIII, The Specific Determination of Proaccelerin, Owren²

The basic principle involved in this test is keeping a reaction mixture constant in thromboplastin, calcium, prothrombin, proconvertin, and fibrinogen.

Proaccelerin-free plasma for the test is obtained by storage of adsorbed ox plasma or from a case of parahemophilia.

Technic.—

The procedure is the same as in the "P and P" method, page 682, using the same concentrations of calcium chloride and thromboplastin with proaccelerin-free plasma.

Comments: This method has been helpful as an investigative tool in the study of clotting mechanisms. By this method, Owren in 1943 was able to detect the first verified case of parahemophilia (factor V deficiency) as a human congenital deficiency. Since then, other cases have been reported as either hereditary or congenital.

These clinical studies have given a new impetus to the study of hemophilia and hemophilia-like diseases.

Method IX, Prothrombin Consumption Method, Quick⁴

Normal Values: 30 to 150 seconds.

Reagents.—

Blood.—

Obtain 6 c.c. of blood by clean venepuncture. Transfer 2 c.c. samples to each of 3 tubes.

Deprothrombinized Rabbit Plasma.—

Mix 9 volumes of freshly drawn rabbit blood with 1 volume of M/10 sodium oxalate. Obtain the plasma by centrifugation.

Measure the plasma after withdrawing it from the blood cells.

For each c.c. of oxalated plasma, place 2.5 c.c. of 0.005 M suspension of tricalcium phosphate in a test tube.

Centrifuge to pack the gelatinous calcium phosphate.

Pour off the surplus water.

Add the required volume of fresh, oxalated plasma.

Mix with the adsorbent.

Stir repeatedly with a small glass rod for 5 minutes at room temperature.

Remove the calcium phosphate by centrifugation, pouring the clear adsorbed plasma into a clean test tube.

¹Alexander, B., Goldstein, R., Landwehr, G., and Cook, C. D.: J. Clin. Invest. 30: 596, 1951.

²Owren, P. A.: Rev. hémat. 7: 147, 1952.

³Owren, P. A.: Am. J. Med. 14: 201, 1953.

⁴Quick, A. J.: Physiology and Pathology of Hemostasis, 1951, Lea & Febiger, Philadelphia, p. 143.

Thromboplastin Solution.—

Incubate 0.2 gm. of acetone-dehydrated rabbit brain with 5 c.c. of physiologic saline at 50° C. for 20 minutes.

Tricalcium Phosphate, 0.005 M.—

Dissolve 158 gm. of trisodium phosphate
in 1,000 c.c. distilled water.

Dissolve 66.6 gm. of anhydrous calcium chloride
in 1,000 c.c. distilled water.

Pour the calcium chloride solution into the trisodium phosphate solution with thorough stirring.

Adjust the reaction to pH 7.0.

Wash the precipitated tricalcium phosphate repeatedly by decantation until the sodium chloride is removed.

Dilute to 1 liter, thus making a 0.2 M suspension.

From this stock, a 0.005 M solution can be prepared by mixing 2.5 c.c. with 97.5 c.c. of distilled water.

0.02 M Calcium Chloride.—**Technic.—**

Place the 2 c.c. samples of blood in a water bath at 37° C.

15 minutes after clotting has occurred, remove tube 1, centrifuge at top speed for 1 minute, and determine the serum prothrombin time by adding 0.1 c.c. of the serum to a mixture of 0.1 c.c. of deprothrombinized rabbit plasma,

0.1 c.c. of thromboplastin, and

0.1 c.c. of calcium chloride solution.

Blow in the serum and time.

Observe for clot formation and record time.

At the end of 30 minutes, centrifuge the second tube, and perform a prothrombin time on this serum in the same manner as in the first tube.

Repeat the prothrombin time on the first tube at this time.

At the end of 45 minutes, recheck the prothrombin times of the sera in tubes 1 and 2.

At the end of 1 hour, centrifuge the third tube and make prothrombin time determinations on the sera of all 3 tubes.

Comment: See comments below on the simplified serum prothrombin test for the basic principles involved.

In this test the serum prothrombin time of normal blood ranges from 30 to 150 seconds. The three tubes tend to show increasing times from the 15-minute test to the 1-hour test. The testing of three tubes in the manner recommended serves as a control over the technic.

In hemophilia, the times vary between 10 and 20 seconds, with very little change in all three tubes. In thrombocytopenic purpura and thrombasthenia, the average time is 25 seconds, and in parahemophilia, it is 34 to 38 seconds.

Method X, Simplified Serum Prothrombin Consumption Test¹

Normal Values: Longer than 30 seconds.

Materials Required.—

Serum obtained from venous blood, and allowed to clot at room temperature. Place the blood in a water bath at 37° C. for 1 hour after clotting.

Centrifuge for 3 minutes to separate the serum. Test the serum immediately or store at 4° C. for 1 hour. Do not use if stored more than 1 hour.

Thromboplastin* Solution.—Prepare as for plasma prothrombin time.

*Simplastin, Warner-Chilcott Laboratories Division, New York.

¹Sussman, L. N., Cohen, I. B., and Gittler, R.: J. A. M. A. 150: 702, 1954.

Fibrinogen Solution*.—This is to contain 300 mg. % of fibrinogen in 0.85% sodium chloride solution.

For Use.—Just before the test, mix 2 c.c. of thromboplastin solution with 1 c.c. of fibrinogen.

Technic.—

Place all reagents in a water bath for 5 minutes.

Place 0.2 c.c. of fibrinogen-thromboplastin mixture in a clean dry test tube.

Add 0.1 c.c. of test serum to mixture by blowing it out of the pipette, simultaneously starting the timer.

Observe for clot formation, and time.

Comment: The basic principle of this test and the Quick prothrombin consumption test is that in the normal process of clotting of blood, prothrombin is almost completely consumed, leaving serum with little residual prothrombin. If there is a deficiency of thromboplastin complex in a blood, prothrombin consumption is diminished, leaving a high prothrombin residual in the serum.

If to these sera are added optimal amounts of fibrinogen and thromboplastin, the time for a clot to form reflects the amounts of prothrombin present. In the case of normal blood, the time is prolonged (beyond 30 seconds). A time of 20 to 30 seconds is considered doubtful and anything below 20 seconds indicative of thromboplastin deficiency.

In thrombocytopenia, whether primary or secondary, the test has yielded low values. This has been true of a case of thrombasthenia. Hemophilia and hemophilia-like diseases give a short serum prothrombin time. When heparin is administered, the serum prothrombin time becomes abnormally low. On the other hand, the coumarins have no effect on the serum prothrombin time since they do not depress thromboplastin complex.

Method XI, Partial Thromboplastin Time (PTT), Langdell et al.¹

Normal Values: Normal values depend on concentration of thromboplastin used and usually are from 40 to 80 seconds.

Materials Required.—

4.5 c.c. blood obtained by clean venepuncture.

M/10 Sodium Oxalate.—

Partial Thromboplastin.—This may be crude cephalin or Asolectin.† The cephalin is prepared as a 0.3% suspension, the Asolectin as a 1% suspension. Adjust by dilution in saline to give a clotting time range of 40 to 80 seconds with normal plasma.

0.02 M Calcium Chloride.—

Technic.—

The procedure is essentially that of the one-stage prothrombin activity test.

Add 4.5 c.c. of venous blood

to 0.5 c.c. of M/10 sodium oxalate.

Separate the plasma by centrifugation.

Keep all reagents at 37° C.

Mix in the following amounts and order:

0.1 c.c. plasma

0.1 c.c. of partial thromboplastin

0.1 c.c. of 0.02 M calcium chloride.

Record the time in seconds for clotting to occur.

*Fibrinogen, Warner-Chilcott Laboratories Division, New York.

†Asolectin is a commercial phosphatide preparation made from crude soya lecithin by Associated Concentrates.

¹Langdell, R. L., Wagner, R. H., and Brinkhous, K. M.: J. Lab. & Clin. Med. 41: 637, 1953.

Comment: The principle is that "partial" thromboplastins give a longer clotting time than "complete" thromboplastins, such as are used in routine prothrombin determinations. Langdell and co-workers found that complete thromboplastins clot normal and hemophiliac bloods equally fast. However, partial thromboplastins clot hemophiliac bloods less rapidly than normal bloods.

This test has been offered as a useful screening procedure in hemophiliac states.¹ It may be substituted for the prothrombin consumption tests which are found on pages 685 and 686.

Sources of Error in Prothrombin Time Estimations.—

1. The venepuncture must be "clean."
2. The ratio between oxalate and blood must not be changed. Excess of oxalate will materially alter the results.
3. In centrifuging the blood, the correct time and speed are 5 minutes at 2,000 r.p.m.
4. Plasma should be removed from the cells as rapidly as possible after centrifuging.
5. Plasma must be kept refrigerated if not handled immediately.
6. The temperature of the water bath must be *exact*.
7. Plasma standing at 37° C. in a water bath loses 50 per cent of its activity within 1 hour.
8. Any detergent used in cleaning glassware must be removed; if left on the glassware, even if only a very faint trace remains, it will interfere with the accuracy of the test.

Comments on Prothrombin Time Determinations

The determination of prothrombin time has become so much a part of daily clinical practice that it is now a household word in the physician's practice. The background development of this interest is typical of how investigations and investigators, great distances apart, are suddenly catalyzed. In fact, a greatly stimulated interest in all phases of coagulation can be said to begin with the epic discovery by Dam,² in 1929, that chicks developed a hemorrhagic syndrome on a synthetic diet.³ By 1935, Dam⁴ reached the conclusion that these chicks were suffering from a vitamin deficiency with a coagulation defect (Köagulation-vitamin or vitamin K). The calendar of events that followed are listed below:

1936: Chicks on artificial diet have prothrombin deficiency.⁵ Quick's quantitative studies on chicks showed prothrombin deficiency; hemorrhage occurred at levels of 10% prothrombin; alfalfa restored chicks to normal.⁶ Quick suggests hypoprothrombinemia of patients with obstructive jaundice is related to vitamin K deficiency.

¹Brinkhous, J. M., Langdell, R. D., Penick, G. D., and Graham, J. B.: J. A. M. A. 154: 481, 1954.

²Owren, P. A.: Am. J. Med. 14: 201, 1953.

³Dam, H.: Biochem. Ztschr. 215: 475, 1929; *ibid.* 220: 158, 1930.

⁴Dam, H.: Biochem. J. 29: 1273, 1935.

⁵Dam, H., Schönheyder, F., and Tage-Hansen, E.: Biochem. J. 30: 1075, 1936.

⁶Quick, A. J.: Am. J. Physiol. 118: 260, 1937.

1937: Quick⁶ reports hypoprothrombinemia and hemorrhagic diathesis in patient with obstructive jaundice. Tried treatment with alfalfa but unsuccessful. Suggested combining bile salts with alfalfa.

1938-1939: Clinical application of Quick's suggestions.⁷

1939: Isolation of vitamin K by Doisy and his associates.⁸

1939-1940: Chemical structure of vitamin K⁹ and its synthesis. Vitamin K_n naphthaquinone.

1940: The race is on for therapeutic use of synthetic vitamin K.¹⁰

This brief sketch of the story of vitamin K obviously does little justice to the many workers who strenuously tried to find answers to the problem before its solution. Many reviews of the story are available and we heartily recommend Quick's review on the hemorrhagic diathesis of avitaminosis K¹¹ as exciting reading. To those of us in the Middle West in the latter part of the 1930's and early 1940's, the excitement of the chain reaction produced by these discoveries reached the feverish pitch of a gold rush. To surgeons everywhere there came a very simple method of controlling the tantalizing and distressing oozing of blood in the patient with obstructive jaundice. The administration of vitamin K to such patients has become so routine as to warrant little further comment. It was quite natural that other clinical states associated with a deficiency of vitamin K be sought for; perhaps the other outstanding group is the hemorrhagic disease of newborn infants. Prophylactic-treatment of the mother just prior to delivery has brought about the same striking results as with the jaundiced patient. It has become routine procedure in obstetrics to administer vitamin K during labor. Lesser in numbers involved are hypoprothrombinemic states associated with vitamin K deficiency due to the following disorders: (1) biliary fistula; (2) poor absorption from the gastrointestinal tract, as in ulcerative colitis, sprue, intestinal fistulae, intestinal obstruction, pellagra, gastrocolic fistula; (3) artificial fever; (4) change of the intestinal flora by sulfonamides; (5) liver disease.

Just as prothrombin determinations may disclose a vitamin K deficiency, serial determinations are helpful in judging the efficacy of vitamin K therapy. If a given clinical state is due to avitaminosis K, administration of the vitamin parenterally or orally should result in a fairly prompt return of prothrombin to normal *provided the liver function is normal*. This fact forms the basis of a liver function test in which standardized doses of vitamin K are administered and prothrombin time determinations are made subsequently.¹¹

⁶Quick, A. J.: J. A. M. A. 110: 1658, 1938.

⁷(a) Bott, H. R., Snell, A. M., and Osterberg, A. E.: Proc. Staff. Meet., Mayo Clin. 13: 74, 1938.

(b) Dam, H., and Glarind, J.: Lancet 1: 720, 1938.

(c) Warner, E. D., Brinkhous, K. M., and Smith, H. P.: Proc. Soc. Biol. & Med. 37: 628, 1939.

⁸(a) Binkley, S. B., MacCorquodale, D. W., Thayer, S. A., and Doisy, E. A.: J. Biol. Chem. 130: 219, 1939; *ibid.* 131: 327, 1939; J. Am. Chem. Soc. 61: 1295, 1939.

(b) Doisy, E. A., Binkley, S. B., Thayer, S. A., and McKee, R. W.: Science 91: 58, 1940.

⁹(a) See 8a, above.

(b) Almquist, H. J., and Klose, A. A.: Am. Chem. Soc. 61: 2257, 1939.

(c) Fieser, L. F.: Science 91: 31, 1940.

¹⁰(a) Almquist, H. J., and Klose, A. A.: J. Am. Chem. Soc. 61: 1923, 1939.

(b) Ansbacher, S., and Fernholz, E.: J. Am. Chem. Soc. 61: 1924, 1939.

(c) Fieser, L. F., Bowen, D. M., Campbell, W. P., Fry, E. M., and Gates, M. D., Jr.: J. Am. Chem. Soc. 61: 1926, 1939.

(d) Thayer, S. A., Cheney, L. C., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A.: J. Am. Chem. Soc. 61: 1932, 1939.

¹¹Quick, A. J.: The Hemorrhagic Diseases, Springfield, Ill., 1942, Charles C Thomas.

It was believed by Lord and Andrus that an increase in prothrombin of 10 per cent or more justified a diagnosis of extrahepatic jaundice. Other workers tried correlating such responses with other liver function tests and, as might be expected, test correlations in liver function followed the traditional course of failing to oblige the investigators. A general principle that appears to have come out of these investigations is that if a patient has hypoprothrombinemia which promptly responds to vitamin K therapy, he does *not* have severe liver damage. The corollary to this is that a poor response indicates either parenchymal liver disease or inadequate dosage.

An interesting situation has been reported in which patients fail to respond to vitamin K therapy and were labeled "idiopathic hypoprothrombinemia" until all the facts became known.

Wright¹ relates the story of a physician with a bleeding diathesis and a prothrombin time of over 200 seconds. A plasma Dicumarol level of 56 to 60 gamma was found, and within a few hours after vitamin K administration there was a prompt response to therapy. The patient was presented with these facts, denied taking Dicumarol, but promptly left Dr. Wright to engage the services of another outstanding coagulationist, Dr. Tocantins. Under the latter's observation, it was noted that the patient asked for 400 to 500 mg. of Demerol daily to control alleged pain. Realizing that his diagnosis of "idiopathic hypoprothrombinemia" was suspect, he left when psychiatric consultation was suggested. Olwin related a similar incident of self-induced hypoprothrombinemia in a nurse, who confessed to overdosing herself with Dicumarol, after being confronted with the facts. The conjectured motives in the physician were the escape of imminent induction into the Army and narcotic addiction. The nurse was probably motivated by a desire for attention. To prove that the meetings of these coagulationists is not all work (and disagreement), Cronkite and Brambel introduced narratives of similar ilk. The members agreed that all cases of "idiopathic hypoprothrombinemia" should be suspect of self-medication. We have had the experience of a patient's being given a prescription of Dicumarol by a physician for a thrombophlebitis. He took 100 mg. daily without returning to his physician, and somehow was able to get his prescription refilled. The result was obvious but fortunately responsive to vitamin K therapy.

As the saga of vitamin K deficiency was exploding, it was not at all surprising that the Wisconsin group under Link's leadership, with the guidance of Quick, promptly solved a mystery plaguing farmers since 1921. Cattle developed a hemorrhagic disease of serious economic proportions which attracted the attention of veterinarians and research men. The events which followed are listed below:

1922-1924: Schofield² attributes disease to spoiled clover ingested by cattle and associated with abnormal blood clotting.

1937: Quick³ demonstrates drop in prothrombin of cattle fed toxic sweet clover hay.

¹Wright, I. S.: Discussion on Tromexan and Dicumarol, Tr. Fifth Conferences on Blood Clotting and Allied Problems, New York, N. Y., Josiah Macy, Jr. Foundation, 1952, pp. 77-78.

²(a) Schofield, F. W.: Canad. Vet. Rec. 3: 74, 1922; J. Am. Vet. M. A. 64: 553, 1924.

³Quick, A. J.: Am. J. Physiol. 118: 260, 1937.

1941: Link and associates* isolate, identify, and synthesize active agents in sweet clover, 3,3'-methylenebis (4-hydroxycoumarin).

1941: Studies on effect of dicoumarin on human beings and experimental animals.† The beginning of Dicumarol therapy.

Since these basic studies, anticoagulant therapy has been extensively used in human disease associated with thrombosis.

Fibrinogen Test, Modification of Andersch and Gibson¹

Reagents.—

0.9% Sodium Chloride Solution, made by dissolving 9 gm. of sodium chloride in distilled water and diluting to 1,000 c.c. with distilled water.

0.25% Calcium Chloride, made by dissolving 250 mg. calcium chloride in distilled water in a 100 c.c. volumetric flask and diluting to 100 c.c. with distilled water.

10% Sodium Hydroxide. Use 10 gm. NaOH per 100 c.c. total volume.

20% Sodium Hydroxide. Use 20 gm. NaOH instead of 10 gm.

Standard Tyrosin Solution. Add 250 mg. tyrosin to about 50 c.c. of N/10 hydrochloric acid in a 100 c.c. volumetric flask and dilute to 100 c.c. with N/10 HCl.

Phenol Reagent of Folin and Ciocalteu (page 305).

Technic.—

Place 25 c.c. of 0.9% sodium chloride solution in a test tube and add 1 c.c. plasma. Mix and add 2 c.c. of 0.25% calcium chloride, placing a small stirring rod in the mixture, and allowing it to stand for one hour. Remove stirring rod containing fibrin and wash with distilled water.

Unknown

Place stirring rod in small tube and add 0.5 c.c. of 10% sodium hydroxide.

Place in boiling water for 30 minutes.

Cool and add 7.5 c.c. distilled water.

Standard

Place 2 c.c. standard tyrosin solution in a large test tube and add

5.5 c.c. of distilled water

and 0.5 c.c. of 10% sodium hydroxide.

Add to both unknown and standard 1 c.c. phenol reagent

and 3 c.c. of 20% sodium hydroxide solution. Mix and read in a colorimeter after 30 minutes.

Calculation.—

$10/R \times 100 \times 0.5 \text{ mg.} \times 13 = \text{mg. fibrinogen in 100 c.c. plasma.}$

Range of fibrinogen is 350 to 750 mg. per 100 c.c. plasma.

Anticoagulant Therapy

Dicumarol (bishydroxycoumarin) (3,3'-methylenebis-4-hydroxycoumarin) has been well established as an adjunct in the management of thromboembolic disease. Perhaps its most universal application in clinical medicine has been in acute coronary thrombosis as a prophylactic against extension of the thrombus. Coronary occlusion has been known to be associated with the formation of mural thrombi which may serve as a nidus for emboli. Prophylactic administration of Dicumarol reduces the incidence of emboli considerably. There is

* (a) Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P.: J. Biol. Chem. 138: 1, 1941.

(b) Campbell, H. A., and Link, K. P.: J. Biol. Chem. 138: 21, 1941.

(c) Stahmann, M. A., Huebner, C. F., and Link, K. P.: J. Biol. Chem. 138: 513, 1941.

† (a) Bingham, J. B., Meyer, O. O., and Pohle, F. J.: Am. J. M. Sc. 202: 563, 1941.

(b) Butt, H. R., Allen, E. V., and Bollman, J. L.: Proc. Staff Meet., Mayo Clin. 16: 388, 1941.

¹J. Lab. & Clin. Med. 18: 816, 1933.

general agreement that dicumarolization reduces the mortality from acute coronary occlusion but some difference of opinion as to what extent. There is sufficient question about the effect on mortality as to reflect on the wisdom of routine use of anticoagulant therapy in myocardial infarction.

Schnur² studied the case records of 1,350 patients with myocardial infarction and found the mortality ranged from 8 per cent in the mildest cases to 95 per cent in the severest cases. He attempted to correlate a great number of variables, giving a rating ("pathologic index") to all cases. He could find no difference in the mortality rates between patients treated with or without anticoagulants, and he found that mortality rates could be predicted on the pathologic index. Furman and associates³ compared a group of 100 patients who received Dicumarol with a group of 211 patients with coronary occlusion who were not treated with anticoagulants. Using various criteria for rating, they established three classes of patients: (a) "good risks," (b) "poor risks," and (c) "very poor risks." In the "good risk" group, there was no significant difference in mortality between those treated with anticoagulants (2 per cent mortality) and those untreated (1.3 per cent mortality). The same was true of the "very poor risks" in whom the mortality rate was over 98 per cent, regardless of treatment. In the intermediate group, "poor risks," there appeared to be some significant difference favoring anticoagulant therapy, with 23 per cent mortality in the treated as compared with 41 per cent mortality in the untreated cases. In this "poor risk group," beneficial results of anticoagulant therapy were greatest in those patients having their first attack of coronary occlusion. Russek and Zohman⁴ sent a questionnaire to several hundred physicians listed in the *Directory of Medical Specialists* relative to the use of anticoagulant therapy in myocardial infarction. Over 50 per cent of those replying to the questionnaire stated that they do not use anticoagulants routinely.

Wright and co-workers⁵ have fully analyzed the findings of a cooperative study of the use of anticoagulants in 1,031 cases of definitely diagnosed coronary thrombosis. Twenty-six per cent of the control subjects developed thromboembolic complications as compared with only 11 per cent in the patients treated with anticoagulants. This included venous thromboses and intracardiac complications as well as cerebral, visceral, and peripheral emboli. They found that thromboembolic phenomena were more likely to occur in the following patients: (1) severely ill, (2) 10 per cent or more overweight, (3) older age groups, (4) those having previous history of thromboembolic phenomena, (5) heart failure or shock, and (6) auricular fibrillation or heart block. These patients are, therefore, particularly in need of anticoagulant therapy. Since no control subgroup showed a lower incidence of thromboembolic phenomena than treated patients, these authors conclude that anti-

²Schnur, S.: *Circulation* 7: 855, 1953.

³Furman, R. H., Ball, C. O. T., Gale, R. G., Billings, F. T., and Meneely, G. R.: *Am. J. Med.* 14: 681, 1953.

⁴Russek, H. I., and Zohman, B. L.: *Am. J. M. Sc.* 225: 18, 1953.

⁵Wright, I. S., Marple, C. D., and Beck, D. F.: *Myocardial Infarction: A Study of 1,031 Cases, Its Clinical Manifestations and Treatment With Anticoagulants*, New York, 1954, Grune & Stratton.

coagulant therapy should be used in "all cases, including mild ones, where positive contraindications do not exist and adequately controlled anticoagulant therapy is feasible."⁶

Wright and co-workers report that mortality statistics do not show as striking a difference as do thromboembolic phenomena when anticoagulant therapy is used, but here again the differences tend to favor the use of anticoagulants. In the control group, 23.4 per cent died as compared with 16 per cent in the treated group. However, thromboembolic phenomena had an incidence of 42 per cent in the control and only 23 per cent in the treated groups that died. If one limits the statistics to the period of potentially effective anticoagulant therapy (the fourth day of therapy through four days after the last dose), only 9.5 per cent of the treated patients died as compared with 17.4 per cent of the control group during a corresponding period. When subclasses were studied, there were found substantial reductions with anticoagulant therapy in fairly much the same groups as those prone to thromboembolic phenomena. "The findings do not support the policy recommended by some of treating only patients appearing severely ill at onset and deferring anticoagulant therapy in milder cases until complications occur."⁷ An important feature is that even though *mortality* in good risk cases is less than 2 per cent, whether treated or untreated, the former showed only nine thromboembolic complications per hundred in contrast with twenty-nine per hundred cases in the latter.

Wright's group comes to the conclusion that all patients with myocardial infarction should receive anticoagulant therapy unless a contraindication to such therapy exists.

Schnur,⁸ in a question-answer address, read in a panel discussion on the use and abuse of anticoagulant therapy, points out that not all of the problems relating to such therapy are readily solved. He particularly indicates the difficulties of the clinical diagnosis of thromboembolism and even questions whether post-mortem examinations can fully reflect this feature or mural thrombi. He believes that the effective ranges of prothrombin at present are at best "educated guesses." His position is that of selective therapy rather than routine. This subject is pursued further by Russek and Zohman⁹ who conclude that "anticoagulant therapy is neither necessary nor desirable for the large segment of patients who sustain their first attack of acute myocardial infarction and present no unfavorable criteria for recovery at the time of the first examination."

Everyone is agreed that the use of anticoagulant therapy is based on a reduction of prothrombin time by Dicumarol and related drugs. Serial determination of the prothrombin time is the only adequate method of control of such therapy and there is no substitute known at present for such control. For this reason, anticoagulant therapy with Dicumarol and related compounds (Tromexan, Link's compound 63, Danilone) should not be considered unless daily prothrombin time determinations are available. The clinical picture, bleeding times, clotting times, and other clinical laboratory procedures for

⁶Modern Concepts of Cardiovascular Disease, Am. Heart A. 23: 208, 1954.

⁷Wright, I. S., et al., loc cit.

⁸Schnur, S.: J. A. M. A. 156: 1127, 1954.

⁹Russek, H. I., and Zohman, B. I.: J. A. M. A. 156: 1130, 1954.

studying the coagulation status of patients are totally inadequate and should not be considered as tools for measuring control of therapy. The purpose of rigid control is twofold; namely, (1) reaching and maintaining effective levels, and (2) avoiding the complication of hemorrhage. Wright and associates found a level of 11 to 23 per cent prothrombin activity as the optimal range for therapeutic effect, and when this range was achieved, thromboembolic phenomena were at their lowest incidence. Prothrombin percentages lower than this range produced no added advantages. Their rule of thumb was a prothrombin time which was two and one-half times the control if the latter were 12 to 18 seconds. If dosage is inadequate and optimal levels are not achieved, benefits will be minimal.

Hemorrhage is the only known complication of Dicumarol therapy and occurs at prothrombin levels of 10 per cent or less. In uncontrolled cases, hemorrhage may be fatal or reach serious proportions. In cases controlled by daily prothrombin times, hemorrhage is generally insignificant. The most common clinical manifestations of hemorrhage are hematuria, hemoptysis, hematemesis, and epistaxis. Bleeding from other sources may occur and in the uncontrolled cases may involve any organ system in the body. The occurrence of hemorrhagic phenomena is a signal for cessation of anticoagulant therapy, careful following of the prothrombin time, and an indication for active treatment to restore the value toward a safe level. The treatment consists of administration of water-soluble vitamin K in a dose of 60 to 72 mg. intravenously once or twice. In the presence of severe bleeding, 250 to 500 mg. of vitamin K₁ oxide orally is recommended. Transfusions of whole fresh blood or plasma may be necessary in cases associated with severe hemorrhage. Transfusions should be considered not only to re-establish the prothrombin status but may be necessary to correct the anemia resulting from blood loss.

There is considerable difference of opinion as to the method of choice in prothrombin determinations. In clinical practice, the aim is relative simplicity with good control. A critical survey of the present knowledge of this procedure leaves us with the impression that oversimplicity is nigh impossible if one is to achieve really good control. The modified dilution methods (modified Quick, Shapiro-Link, or the "P and P" test of Owren) appear to answer the problem of adequate laboratory control. The literature covering methods and materials is so voluminous that a review of this aspect alone would be impossible here. Suffice it to say that whatever technic is employed, certain basic principles must be adhered to; namely, (1) that the technic be followed scrupulously as directed by the author of the technic, (2) materials be assayed carefully for potency, and (3) controls be included in any determination. Perhaps some of the differences in clinical results are reflections of poor laboratory control rather than an index of failure of anticoagulant therapy. One objection to insistence on such high standards of laboratory control is that hospitalization becomes almost imperative in order to achieve this. The resultant economic strain on hospitalized patients is used as an argument against routine anticoagulant therapy. However, in disease as serious as coronary occlusion, it is somewhat difficult to argue against this care, except perhaps in the mildest cases. Wright¹⁰ argues against withholding anticoagulants be-

¹⁰Wright, I. S.: *Circulation* 5: 161, 1952.

cause it is impossible to predict which cases might develop thromboembolic phenomena. He states that "unfortunately such prophetic vision foresight has failed. It is more difficult to predict whether the course of a case will be mild or severe on the day of onset, when decisions regarding therapy must be made, than in retrospect, after the patient has been discharged from the hospital. If a physician waits until the first thromboembolic episode occurs, he may have a permanently disabled patient, as in the case of a hemiplegia due to an embolus to the brain, or a dead one."

Dicumarol dosage is determined by routinely estimating the prothrombin activity prior to the initial dose. If the prothrombin time test is relatively normal, then the following dosage schedule is recommended¹¹:

First day: Give one dose of 200 to 300 mg. orally, depending on the size and condition of the patient.

Second day: Determine prothrombin time and if activity is more than 25 per cent, give 100 to 200 mg. of Dicumarol.

Third and each subsequent day: Determine prothrombin time and administer dosage based on previous experience with the patient. If the prothrombin activity is greater than 25 per cent, administer 50, 100, or 200 mg. as previous response indicates.

Wright recommends continuation of therapy for approximately 4 weeks in patients with coronary occlusion. If for any reason Dicumarol therapy has been discontinued and there occurs an indication for re-instituting therapy, treatment must be based on the same controls as originally used. Dosages are guided by the patient's previous response and daily prothrombin activity determinations. There is apparently no sensitivity to the drug, and resistance occurs only if vitamin K₁ therapy has been recently used. Either larger doses of Dicumarol may be necessary or heparin may have to be added to the anticoagulant regimen. If patients on anticoagulants require surgery for any reason whatsoever, the prothrombin time should be returned to normal before surgery to avoid hemorrhage.

In addition to the use of anticoagulant therapy in coronary thrombosis, it is recommended in the following conditions:

1. Majority of cases of thrombophlebitis regardless of origin, including recurrent idiopathic thrombophlebitis, post-infectious, or post-traumatic. Wright¹² makes the interesting observation that a thrombophlebitis that progresses relentlessly despite adequate therapeutic levels of the coumarin compounds should arouse the suspicion of malignancy. He cites 20 examples of persistence of migratory phlebitis refractory to adequate therapy. Carcinoma of the pancreas and liver are most commonly associated with distant thrombophlebitis, although other organs may also have such association.

2. The prevention of postoperative thromboembolic disease. Although statistics generally show a lower incidence of postoperative thrombophlebitis or embolus¹³ in patients given prophylactic dicumarolization, there is some

¹¹Brochure on package information, Abbott Laboratories, North Chicago, Ill.

¹²Wright, I. S.: *Circulation* 5: 161, 1952.

¹³Wise, W. D., Loker, F. F., and Brambel, C. E.: *Surg., Gynec. & Obst.* 88: 486, 1949.

question as to the wisdom of such procedure *routinely*, and perhaps it should be reserved for selected cases in which there is a notoriously high incidence of postoperative thrombophlebitis, such as pelvic surgery.

3. Patients with acute embolic or thrombotic occlusion of the peripheral arteries from any of several causes.

4. Patients with recurrent embolization and auricular fibrillation, such as occurs in rheumatic heart disease.

5. Post-partum patients with previous histories of recurrent thrombophlebitis.

Dicumarol therapy should be used with caution in the following conditions:

1. Severe malnutrition and cachexia.

2. Unusually prolonged menses or menorrhagia.

3. Patients with reduced prothrombin activity due to salicylates or para-aminosalicylic acid in large doses. If Dicumarol therapy appears of greater value in treating a particular patient, it is recommended withholding large dosages of these drugs which can of themselves reduce the prothrombin activity.

4. Patients under prolonged antibiotic or sulfonamide therapy in which changes in the intestinal flora affect vitamin K synthesis.

5. Severe hypertension.

6. In any patient with an initial lowered prothrombin activity unless the cause for such lowering can be well delineated and in itself does not constitute a contraindication to anticoagulant therapy.

7. In patients recently subjected to operations on the central nervous system.

8. In patients with fever. Animal experiments have shown an accentuated response in the presence of fever.

The contraindications to anticoagulant therapy are:

1. *Inadequate facilities for prothrombin determinations.*

2. Patients with hemorrhagic diatheses, particularly where prothrombin is affected directly or indirectly as a result of the disease. Hemophilia-like syndromes and the thrombocytopenic purpuras are obvious examples of such diseases. In the thrombopenic states, the lowered platelet count may be primary or secondary. It is safe to say that any patient showing a defect of the coagulation mechanism should be eliminated from anticoagulant therapy.

3. Pregnancy in the second or third trimester.

4. Patients scheduled for immediate surgery.

5. Patients with moderately or fully advanced renal or liver disease.

6. Patients with jaundice due to biliary obstruction or hepatocellular disease.

7. Patients under continuous tube drainage of the stomach or small intestine.

8. Patients with hemorrhage of the central nervous system.

9. Patients with ulcerating lesions (gastrointestinal tract) and granulomatous lesions.

Since the introduction of anticoagulant therapy with Dicumarol, other drugs have been found and studied. Tromexan or 3,3'-carboxymethylene bis-(4-hydroxycoumarin) ethyl ester is a recent derivative and, as its chemical structure indicates, related to Dicumarol. Compound 63 of Link¹⁴ is a 4-hydroxycoumarin which has also been used in man and animals. Another group of anticoagulants which have come into more recent use are indanedione derivatives, such as Danilone* (2-phenyl-1,3-indanedione) and Indon† (2-phenyl-1,3-indanedione). These two related compounds have a more rapid effect on the prothrombin activity both in lowering the level and in returning it to normal after cessation of therapy. The hypoprothrombinemia induced by them is more refractory to vitamin K therapy than the coumarin derivatives. Excellent comparative studies of these newer anticoagulants are to be found in the *Fourth and Fifth Conferences on Blood Clotting of the Josiah Macy, Jr., Foundation*.¹⁵

Heparin and Postoperative Thrombosis and Embolism.—Crafoord and Jorpes of Stockholm, Sweden, utilized heparin in attempting to prevent postoperative vascular thrombosis. In 325 patients treated with heparin, no great complication occurred, whereas, in the control series of 1,111 similar cases, complications occurred in 9 per cent. In Sweden, 30,000 doses of heparin of 50 to 100 mg. each have been given intravenously without any fatalities or any noteworthy drawbacks.

Murray and Best found no thrombosis or embolism in 400 patients. Moreover, patients with phlebitis were improved by treatment with heparin, and favorable effects of heparin in a small number of cases of pulmonary embolism were reported.

Heparin is an anticoagulant used alone or in conjunction with the aforementioned anticoagulants. Its action is a direct one on the coagulation mechanism and does not affect the prothrombin activity of blood. Its action is followed by determination of the clotting time and not by prothrombin time determinations.

BLOOD FINDINGS IN VARIOUS DISEASES

THE ANEMIAS

The term "anemia" is a clinical conception, which was used originally to designate a decrease in the total quantity of blood. It is now applied much more frequently to diseases accompanied by a decrease in the number of erythrocytes or hemoglobin content or both.

Classification of the Anemias

Wintrobe¹⁶ grouped the anemias on the basis of the absolute constants of the red cells and hemoglobin. This classification is as follows: (1) macrocytic anemia; (2) normocytic anemia; (3) simple microcytic anemia, and (4) hypochromic anemia.

*Schieffelin & Co., New York.

†Parke, Davis & Co., Detroit.

¹⁴Ikawa, M., Stahmann, M. A., and Link, K. P.: J. Am. Chem. Soc. 66: 902, 1944.

¹⁵Blood Clotting and Allied Problems, J. E. Flynn, ed. Tr. Fourth Conf. New York, Josiah Macy, Jr., Foundation, 1951. Blood Clotting and Allied Problems, J. E. Flynn, ed. Tr. Fifth Conf. New York, Josiah Macy, Jr., Foundation, 1952.

¹⁶Wintrobe, M. M.: Proc. Soc. Exper. Biol. & Med. 27: 1071, 1930.

TABLE 52.—THE ANEMIAS*

TERMS TO BE USED	TERMS TO BE AVOIDED
<i>Normocytic anemia</i> , due to Unknown cause <i>Hypoplastic normocytic anemia</i> (pancytopenia), due to Unknown cause	Refractory anemia, secondary anemia
Congenital Poison (specify, as benzol) Infection Radiation, from Roentgen rays Radioactive substances Osteopetrosis Myelofibrosis Congestive splenomegaly, due to Unknown cause Thrombosis of splenic or portal vein Liver disease	Idiopathic aplastic anemia, panmyelophthisis, aregeneratory anemia, aleukia haemorrhagica, toxic paralytic anemia, cryptogenic anemia, refractory anemia, pancytopenia, Felty's syndrome Fanconi's syndrome, constitutional infantile anemia
<i>Normocytic anemia with erythrocytic hypoplasia</i> , due to Unknown cause	Simple chronic anemia, secondary anemia
Congenital Endocrine hypofunction Hypothyroidism Poison Infection	Marble bone disease, Albers-Schönberg disease
<i>Normocytic anemia with myelophthisis</i> , due to Unknown cause Osteopetrosis	Banti's disease, splenic anemia
Myelofibrosis Amyloidosis Metastatic neoplasm Leukemia Plasmacytic myeloma	Pseudoaplastic anemia, refractory anemia, progressive hypocythemia, progressive erythrophthisis
<i>Normocytic anemia from internal destruction of erythrocytes</i> , due to Unknown cause Poison Lead Infection Parasite Malaria Sensitization to Rh-Hr or other erythrocytic agglutinogens Hemolytic disease of the (fetus and) newborn Transfusion hemolysis Internal hemorrhage Hemoglobinuria Unknown cause Paroxysmal hemoglobinuria Unknown cause Nocturnal Cold Exertional Allergic Favism	Leuko-erythroblastosis, myelopathic anemia, metastatic anemia Osteopathia condensans disseminata, chronic non-leukemic myelosis, Albers-Schönberg disease, marble bone disease
<i>Hemolytic normocytic anemia</i> , ac-	Hemolytic anemia
	Erythroblastosis fetalis, erythroleukoblastosis, icterus gravis neonatorum, hydrops fetalis
	Hemoglobinemia
	Marchiafava-Micheli syndrome "e frigore" March hemoglobinuria

*Reproduced from Am. J. Clin. Path. 20: 562-571, 1950, by courtesy of the Editor and of the Williams and Wilkins Company, Baltimore.

TABLE 52.—CONT'D

TERMS TO BE USED	TERMS TO BE AVOIDED
quired due to Unknown cause Acute	Lederer's anemia, Winckel's disease, acute febrile pleiochromic anemia
Cold agglutinins Sulfonamide sensitivity Leukemia, lymphocytic Ovarian cyst Hemolytic normocytic anemia, hereditary, due to Hereditary spherocytosis	Familial hemolytic icterus, acholuric jaundice, congenital hemolytic icterus, hemolytic icteroanemia
Trait Hereditary ovalocytosis Trait Sicklelemlia	Sicklanemia, drepanocytic anemia, Dresbach's syndrome, Herrick's anemia
Trait Normocytic anemia, due to Acute blood loss Metabolic disturbance, from Unknown cause Lipid histiocytosis Protein deficiency Vitamin deficiency Sprue	Secondary anemia, chlorosis
<i>Hypochromic microcytic anemia</i> , due to Unknown cause	Idiopathic anemia, simple achlorhydric anemia, Witt's anemia, chronic microcytic anemia, idiopathic hypochromaemia, chronic chlorosis, erythronormoblastic anemia
Chronic blood loss Deficient intake, absorption, or metabolism of iron Prematurity Hereditary leptocytosis	Secondary anemia, hypoferric anemia, achromic anemia, simple anemia, iron deficiency anemia
Trait With erythrocytosis Pernicious anemia, type <i>macrocytic anemia</i> , due to Unknown cause	Cooley's anemia, thalassemia, erythroblastic anemia, Mediterranean anemia, disease, or fever, target cell anemia, familial microcytic anemia, hereditary or familial poikilocytosis
Infantile Pernicious anemia	Achrestic anemia, idiopathic refractory megaloblastic anemia
Pregnancy	Megaloblastic anemia of infancy Addisonian anemia, Biermer's disease, primary anemia, macrocytic anemia, hyperchromic anemia, megalocytic anemia, megaloblastic anemia, "Severe" anemia of pregnancy, primary anemia of pregnancy, hemolytic anemia of pregnancy, macrocytic anemia of pregnancy
Sprue Tropical	Indische Sprue, psilosis, aphthae tropicae, tisis intestinal, Cochín-China diarrhoea
Nontropical Nutritional deficiency Celiac disease Chronic intestinal obstruction Diphyllobothriasis Gastrectomy Disturbance in continuity of the gastrointestinal tract Tuberculous enteritis	Idiopathic steatorrhea, Gee-Herter disease Goat's milk anemia, tropical macrocytic anemia
<i>Macrocytic anemia</i> , due to Unknown cause Hypoplasia Liver disease	Achrestic anemia

On this basis of classification, under the macrocytic normochromic anemias would be included pernicious anemia, sprue, and the so-called pernicious anemia of pregnancy. The normocytic normochromic anemias would include those which follow acute hemorrhage and aplastic anemia. The microcytic normochromic anemia is the type that occurs in various infections and intoxications, while under hypochromic microcytic anemia there would be included the anemias in which there is marked chronic blood loss or iron deficiency.

Anemias may be due to disturbances in the bone marrow function. **Acute loss of blood** produces an anemia which persists until restitution through active bone marrow function. First there occurs in the circulating blood a flooding of young forms, and perhaps a leukocytosis due to the proliferation of the myeloid system. There is, of course, present in the blood of these individuals after an acute loss of blood hemoglobin-poor immature red cells. There is a lowered color index. There is necessarily a reparative polycythemia. Erythropoiesis continues, the cells approach closer to the normal, and the red blood cell count, which has been elevated by reason of the accelerated bone marrow function, becomes lower, and the color index approaches 1.

As a result of infectious diseases, the bone marrow function is disturbed by the toxin. This is followed by an anemia. There is an anemia seen in typhus, polyarthritis, and syphilis.

A more intense anemia is seen in connection with **malignant tumors**. We find a very severe grade of anemia in carcinoma of the esophagus and stomach. **Chemical substances**, such as lead, mercury, nitrobenzene, benzol, when ingested all produce an anemia.

We have elsewhere alluded to the basophilic punctation seen in lead poisoning. This condition is the result of the toxic effect upon bone marrow erythropoiesis. Further etiological factors in anemia are **intestinal parasites**, such as *Diphyllobothrium latum*, *Taenia*, and *Trichuris*. *Ancylostoma duodenale* and *Necator americanus* produce anemia by loss of blood.

Discussion of anemia due to the *Diphyllobothrium latum* has been carried on in medical literature for some time. The question that has agitated the writers on this subject is whether or not this fish tapeworm in disease causes the anemia which is so like a case of pernicious anemia or whether, as Magath² has stated, the presence of *Diphyllobothrium* is considered only as a "trigger" that sets off the illness. Schauman,³ the leading European investigator of this problem, maintains that *Diphyllobothrium* is capable of causing an anemia which is not distinguishable from true primary pernicious anemia, and that the reason the incidence of the disease is so small is that the "ordinary host is refractory toward the anemia-causing agent of the tapeworm." Piney⁴ has called attention to the close resemblance between this anemia and true primary pernicious anemia. He believes that this condition may be elicited in some by the toxins of the fish tapeworm but thinks a constitutional predisposition probably exists in these patients. Birkeland⁵ reviewed all the foreign as well as the American literature on *Diphyllobothrium* anemia, and,

²Magath, T. B.: J. A. M. A. 101: 337, 1933.

³Schauman, O.: Die perniciose Anämie im Lichte der modernen Gifthyphothese, Samml. klin. Vortr. pp. 231-282, 1900. Schauman, O., and Saltzman, F.: Die perniziose Anämie, in A. Schittenhelm's Handb. d. Krankh. des Blutes und der blutbildenden Organe, Berlin, Julius Springer 2: 100-258, 1925.

⁴Piney, A.: Recent Advances in Hematology, Philadelphia, 1927, P. Blakiston's Son & Co., p. 71.

⁵Birkeland, I. W.: Bothriocephalus Anemia, Medicine 11: 1, 1932.

after presenting the observations and opinions of apparently all who have written on this subject, in an unbiased but critical manner, concluded that the anemia occurring in symbiosis with an infestation by *Diphyllobothrium* is probably not due to any specific substance elaborated by this parasite but to a constitutional factor which would probably cause this type of patient to have a pernicious anemia without the presence of the parasite, granting that there occurs some unknown precipitating factor in this disease. In other words, he considers patients who suffer and survive *Diphyllobothrium* anemia as having had an abortive form of true primary pernicious anemia. Von Bonsdorff⁶ suggests that the pernicious anemia picture of *Diphyllobothrium*

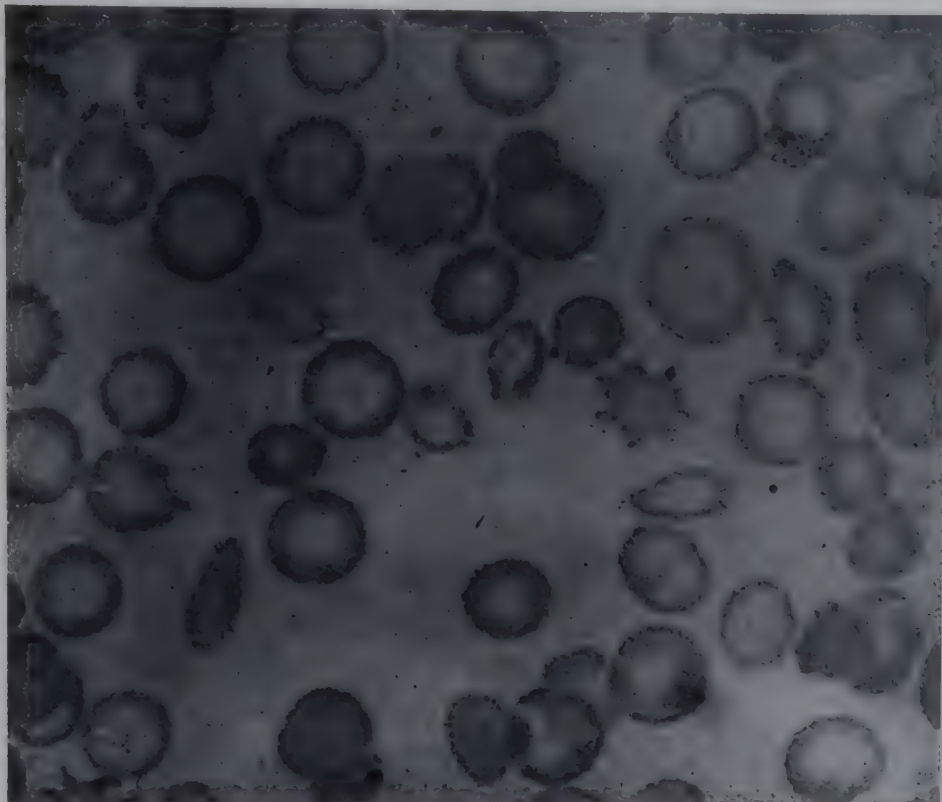


Fig. 178.—Blood film, hypochromic anemia, showing hypochromia, poikilocytosis, and anisocytosis. ($\times 950$.)

infestation is due to two possible mechanisms. One involves the competition of parasite and host for vitamin B₁₂; in the event that the worm wins in this race, a deficiency develops in the host. An alternative, or contributory mechanism, is that intrinsic factor production may be diminished if the parasite is located high up in the small intestine. If the parasite moves lower down in the gastrointestinal tract, the pernicious anemia picture disappears.

Isaacs, Sturgis, and Smith studied a patient with a fish tapeworm infestation who had a typical blood and clinical picture of primary pernicious anemia which was amenable to liver therapy before removal of the tapeworm and who had no relapse of the anemia following the eradication of the parasite, even when liver therapy was stopped. Seyderhelm has demonstrated that the ground-up body of the fish tapeworm has a hemolytic action both in vitro and in vivo. Hunnicutt⁷ has given a report on a very interesting case of a native of Finland who had a disease which was singularly like true pernicious anemia and cleared up under liver therapy and who later showed

⁶Von Bonsdorff, B.: *Acta haemat.* 10: 129, 1953.

⁷Hunnicutt, Thos. N., Jr.: *J. A. M. A.* 104: 1984, 1935.

eggs and segments of the worm. After suitable therapy the worm was eliminated and the patient was discharged with a normal blood count; he remained normal after fourteen months, without a return of the anemia and with no specific therapy during this period. It would seem, according to this writer, that this was probably a case of a true tapeworm anemia.

Enterogenous anemias, due to **infectious septic processes**, particularly ulcerative colitis, produce a severe toxic anemia. **Chronic pyelitis** also produces severe anemia.

Hemolytic substances, such as potassium chlorate, purine, etc., when ingested, so disturb the red cells in the blood stream that there develops a hemoglobinemia and a hemoglobinuria. Attention must be called, too, to the high-grade anemia seen in **nephritis**. Nonnenbrouch⁸ believes this is due to hydremic plethora.

The "**proletariat**" anemia of Naegeli is a simple anemia traceable to lack of food. There is another type of simple anemia due to climatic conditions, the so-called "**tropical anemia**."

The **influence of light** upon the causation of anemia has been a subject of discussion. It is improbable that it has any effect. For instance, the long polar night, according to Nansen's figures, did not produce anemia as long as the nutritional state of the explorers was good.

Schoenenberger and Semple state that horses which have been kept underground ten to twenty years in mining and tunnel operations show no anemia.

Naegeli has shown that there are anemias produced through **disturbances in the internal secretory apparatus**.

Later there will be discussed some other forms of anemia referable to children (see Remarks on Hematology of Infants and Childhood).

Constitutional Hemorrhagic Anemia has been described by Gaesslen. In these cases, there are disturbances in development of the mandible, fingers, hands, sometimes microphthalmus, internal deformities, sclerosis, and muscle dystrophy. In these cases, there is usually a megalosplenism. This disease has baffled hematologists as to its origin. It is noteworthy that the children of these constitutional hemolytic anemic patients show the same disease, down to the third generation. Such patients show constitutional changes in the red cells and a marked change in the fragility test.

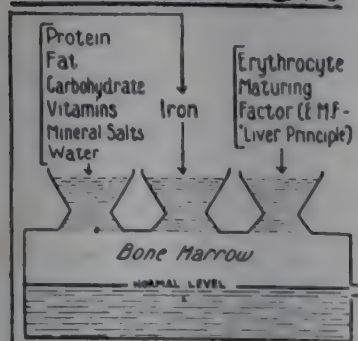
Idiopathic Hypochromic or Microcytic Anemia is a new development in the study of hematology. It is characterized by an absence of free hydrochloric acid in stomach contents in middle-aged women who have a microcytic type of red cell. These patients respond to iron just as the chlorotic patients do. The condition simulates chlorosis except that it occurs in middle age and there is no hydrochloric acid in the gastric contents. For further discussion of this very interesting condition, see the review by Wintrobe and Beebe.⁹

Figs. 179 to 191 by Haden graphically present the state of the bone marrow, peripheral blood, and related organs with their relative status in normal and various abnormal conditions related to the red blood cells. Study of these illustrations is highly remunerative when related to the discussion in the text.

⁸Nonnenbrouch: Arch. f. exper. Path. 91: 218, 1921.

⁹Wintrobe, M. M., and Beebe, R. T.: Idiopathic Hypochromic Anemia, Medicine 21: 187, 1933.

FORMATION

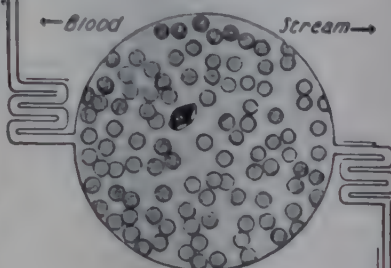


NORMAL ADULT

Red cells formed largely in ribs, skull and vertebrae
Total normal volume of marrow is 1400 cc. Normal color is greyish red
For red cell formation, non-specific substances necessary for all cells are required as well as two specific substances, iron and the erythrocyte maturing factor (E.M.F.)
One trillion cells and 25 gms HB formed each day
Rate of formation measured by reticulocyte percentage in circulation
Normal life of red cell about 30 days

CIRCULATION

Twenty-five trillion red cells in blood stream. Each cell during its life, makes 50,000 to 100,000 complete circuits from lungs to tissues. Functions as conveyor for hemoglobin which carries oxygen from lungs to tissues
Blood count represents balance between red cell and hemoglobin formation and destruction

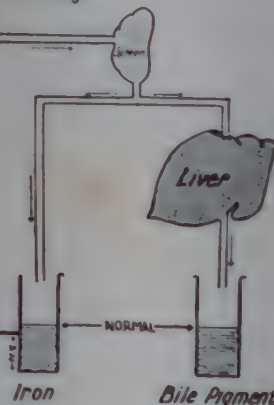


EXAMPLE

RBC 50 Million
HB 100% (134 gms)
Volume Index 100 (90 cubic microns)
Color Index 100 (32 micromicrograms)
Saturation Index 100 (34.7%)
Icterus Index 46 units
Reticulocytes 0.5-10%

DESTRUCTION

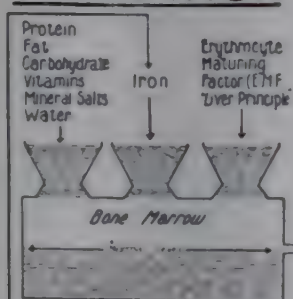
Old red cells taken out by reticulo endothelial cells, largely those of spleen
1 trillion red cells destroyed each day
25 gms HB destroyed each day
100 mg. of iron released
65 mg. used again
15 mg. lost and replaced by food
500 cc. bile formed and excreted by the liver containing 300 to 400 mg. of bile pigment
Rate of destruction gauged by iron released and bile pigment formed
Only clinical laboratory measure is icterus index or quantitative Van den Bergh



Eighty-five percent of iron released returns to marrow to be used again

Fig. 179A.—Normal red cell physiology. The cells are being formed and destroyed at a constant rate. The cells vary little in volume and hemoglobin content. The rate of delivery of new cells is gauged by the reticulocyte count and the rate of destruction by the icterus index. The blood count and hemoglobin content show only the balance between the formation and destruction of red cells. *Editor's note:* Normal life of the red blood cells is 120 days instead of 30 days. (From Haden: Principles of Hematology, Lea & Febiger. Modified from Haden: J. Lab. & Clin. Med.)

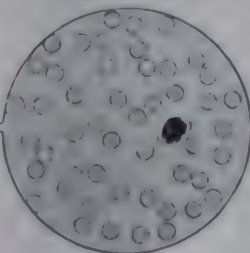
FORMATION



No immediate change in marrow
Later becomes more active

CIRCULATION

← Blood Stream →



EXAMPLE

RBC 250
HB 50%
Volume Index 100
Color Index 100
Saturation Index 100
Icterus Index 3
Reticulocytes 1%

DESTRUCTION

Rate of hemoglobin destruction is immediately normal. Later is decreased

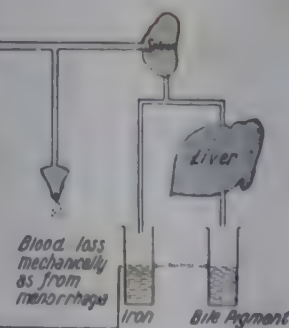


Fig. 179B.—The red cells after an acute hemorrhage. The cells are reduced in number but normal in size (volume index 1), and hemoglobin content (color index 1). (From Haden: Principles of Hematology, Lea & Febiger. Modified from Haden: J. Lab. & Clin. Med.)

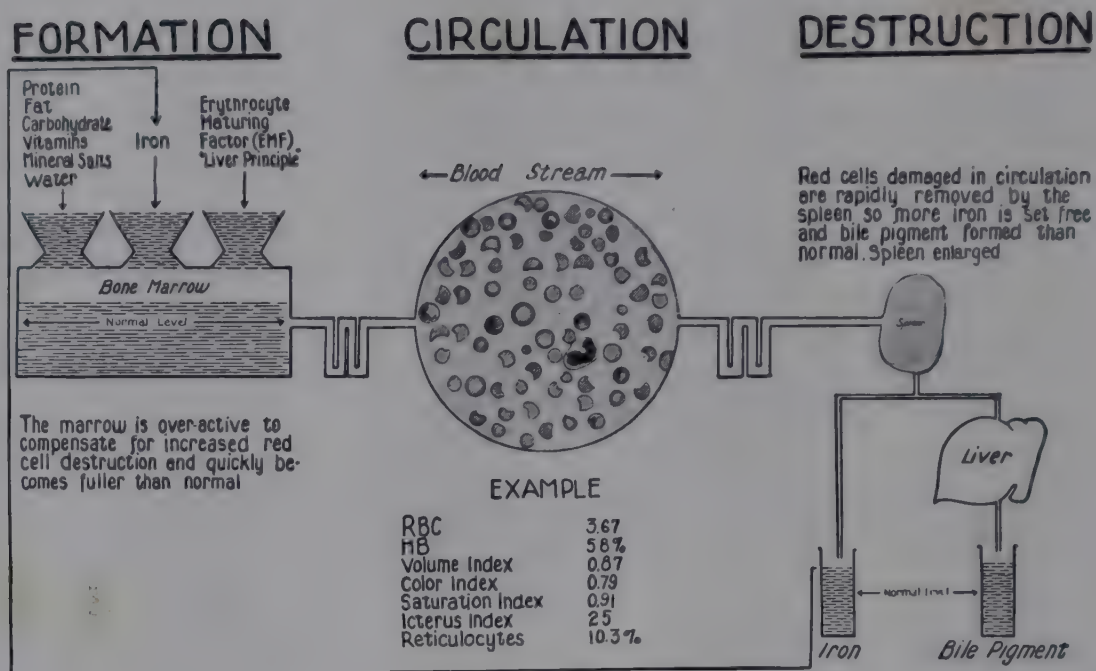


Fig. 180.—The red cells in excessive destruction by a toxic substance (phenylhydrazine). The red cells are damaged and then rapidly removed from the circulation by the spleen so splenomegaly results. The high icterus index (25 units) is due to the accelerated destruction of hemoglobin. The high reticulocyte count (10.3 per cent) is an attempt on the part of the bone marrow to compensate for the abnormal destruction of cells. (From Haden: Principles of Hematology, Lea & Febiger. Modified from Haden: J. Lab. & Clin. Med.)

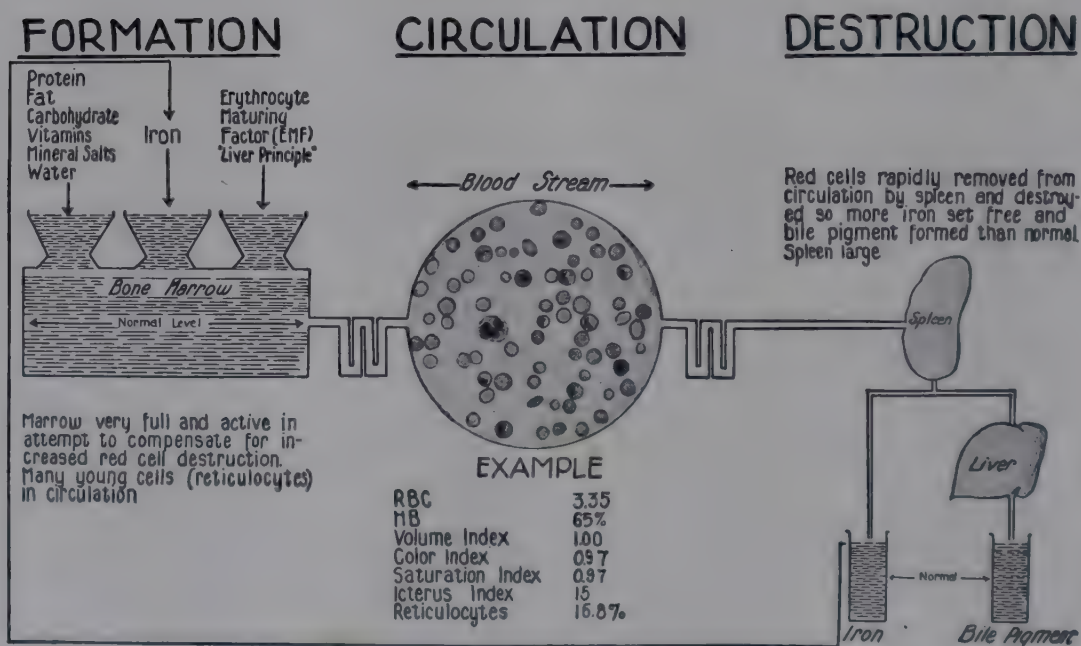


Fig. 181.—The red cells with excessive removal from the blood stream by the spleen because of spherocytic shape. In congenital hemolytic icterus the cells are abnormal in shape (spherocytes) and are rapidly removed from the circulation. The icterus index is high (15 units) due to the excessive destruction and the reticulocyte per cent is also high (15.8 per cent) indicating an excessive release of red cells from the marrow. In this condition the cells remain in the circulation only a few days as compared with a normal time of 120 days. (From Haden: Principles of Hematology, Lea & Febiger. Modified from Haden: J. Lab. & Clin. Med.)

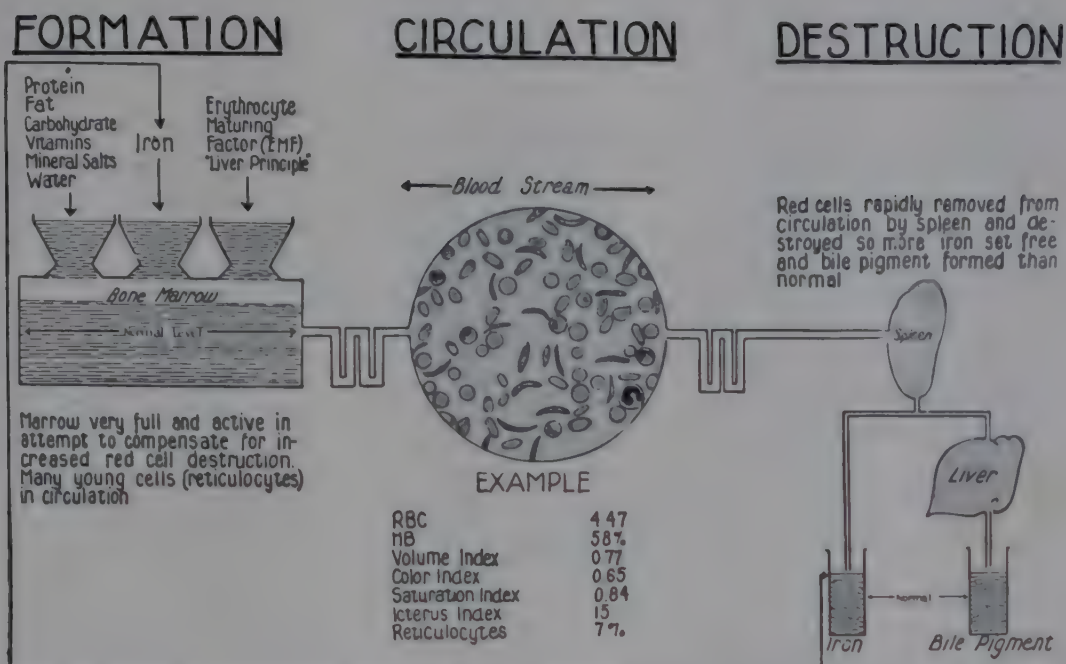


Fig. 182.—The red cells in excessive removal from the blood stream by the spleen because of sickle shape. This is also a hemolytic anemia as shown by the increased icterus index and the reticulocytes. (From Haden: Principles of Hematology, Lea & Febiger. Modified from Haden: J. Lab. & Clin. Med.)

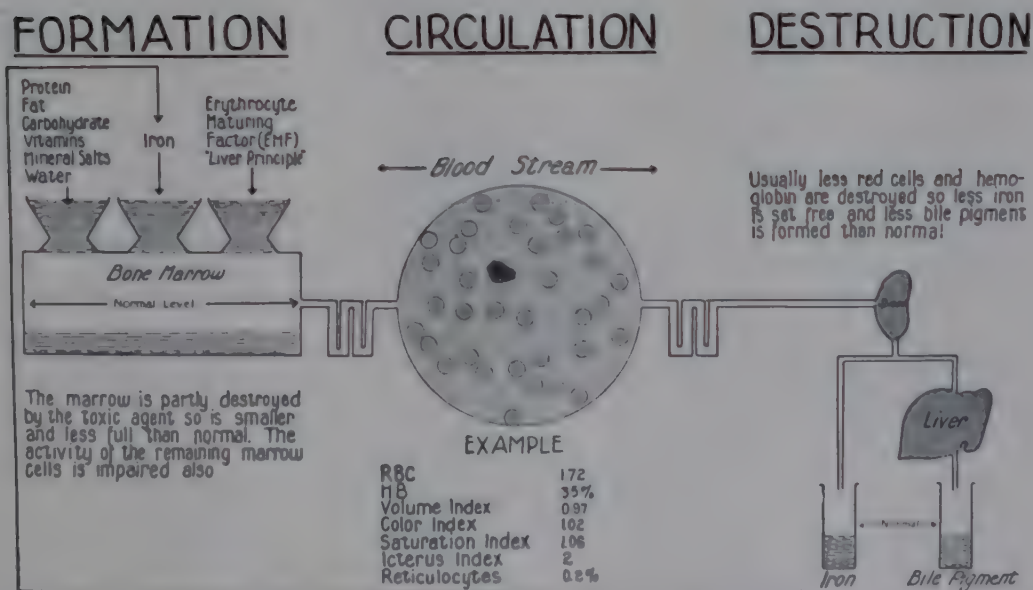


Fig. 183.—Red cells in anemia due to aplasia of marrow by myelotoxic agent as arsphenamine. Here the marrow is decreased in volume and few erythrocytes, leukocytes, or thrombocytes can be delivered. Such cells as are found are normal. (From Haden: Principles of Hematology, Lea & Febiger. Modified from Haden: J. Lab. & Clin. Med.)

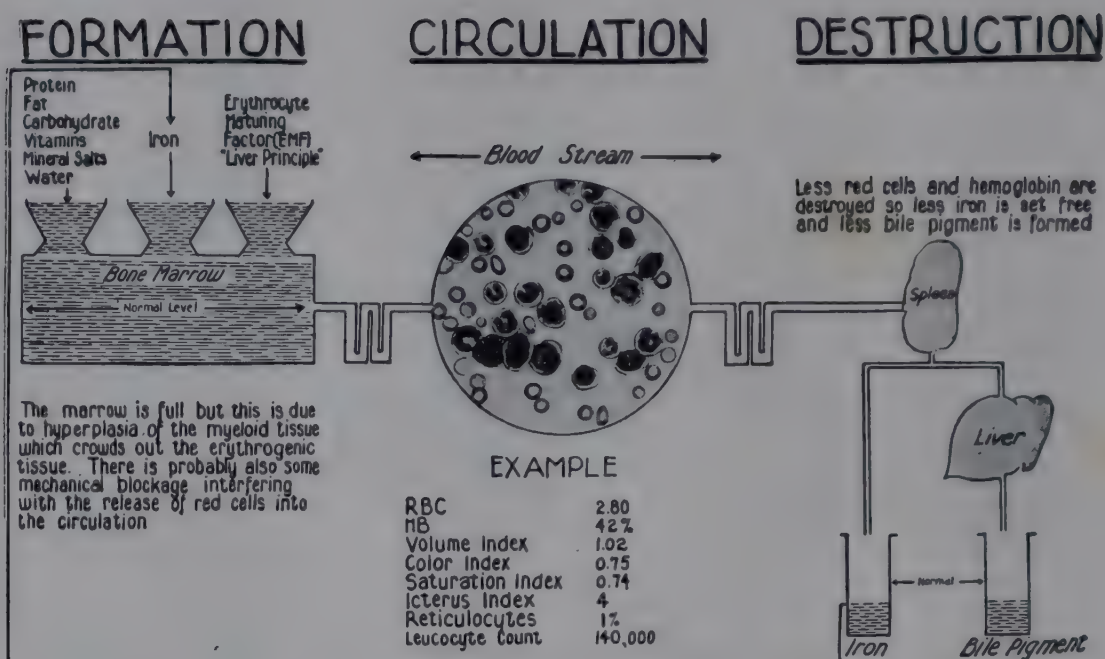


Fig. 184.—The red cell in a quantitative depression of the bone marrow as by leukemia. Here the erythropoietic tissue is crowded out by the leukopoietic tissue so less cells than normal can be formed. Such cells as are formed are normal in volume and hemoglobin content. (From Haden: Principles of Hematology, Lea & Febiger. Modified from Haden: J. Lab. & Clin. Med.)

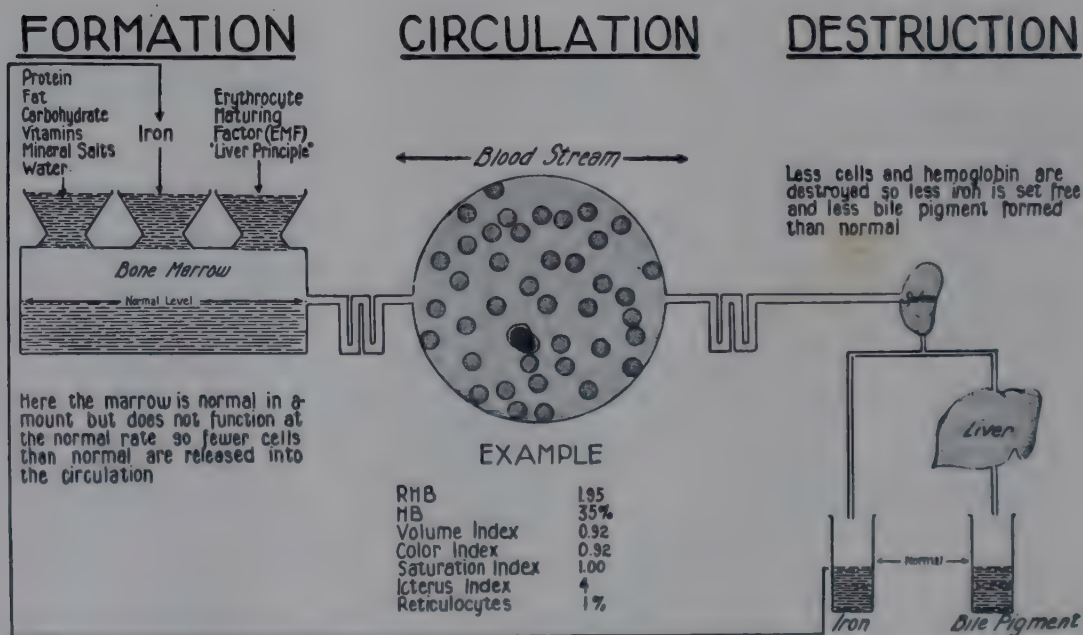


Fig. 185.—The red cell in a quantitative depression of the bone marrow as by nephritis. Here the amount of functioning tissue is not decreased but the rate of activity is much less than normal. Again the cells formed are normal in volume and hemoglobin contents. (From Haden: Principles of Hematology, Lea & Febiger. Modified from Haden: J. Lab. & Clin. Med.)

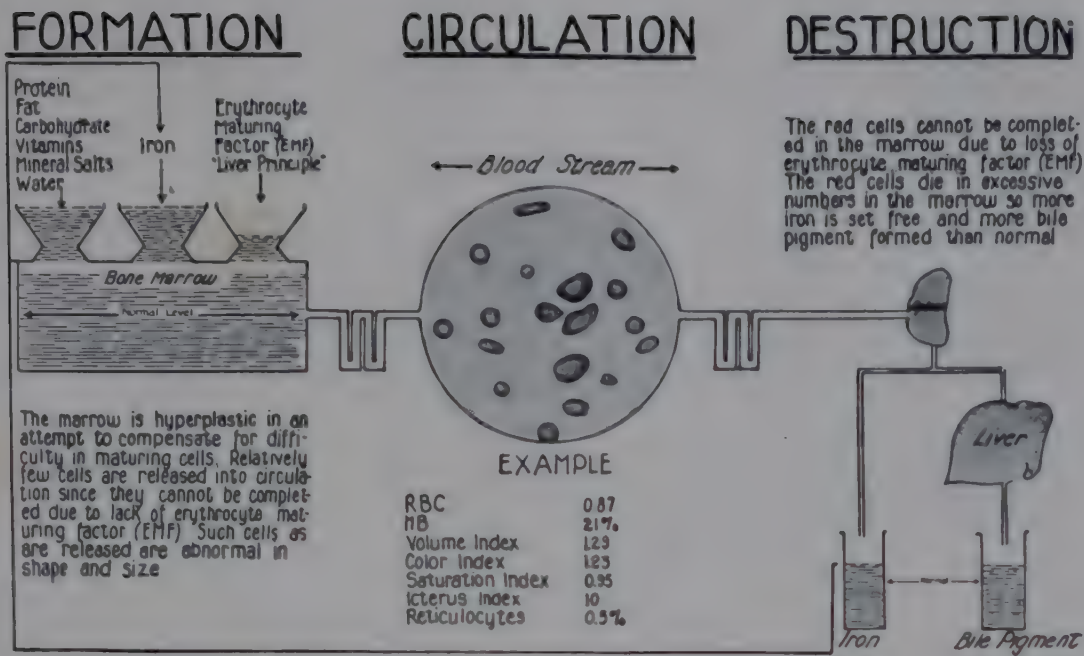


Fig. 186.—Red cells in an anemia due to an idiopathic deficiency in the erythrocyte-maturing factor (pernicious anemia). Here the marrow is hyperplastic because the cells which cannot be normally completed accumulate. Such cells as are released are not normal as shown by the macrocytosis (volume index 1.29) which indicates a deficiency of the specific-maturing factor (EMF). Many cells die in the marrow, since they are not normally released, so the bile pigment content is increased (icterus index, 10 units). (From Haden: Principles of Hematology, Lea & Febiger. Modified from Haden: J. Lab. & Clin. Med.)

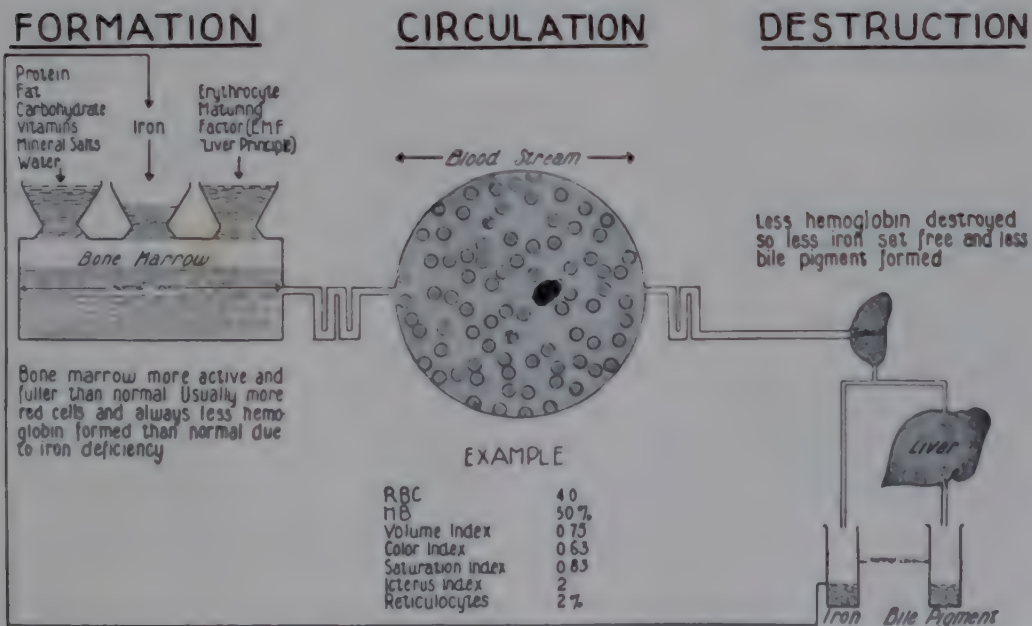


Fig. 187.—The red cells with a deficient intake of iron. As a result of the chronic iron deficiency, the hemoglobin is reduced (color index 0.63) and the cells become smaller (volume index 0.75). The icterus index is low because less hemoglobin is destroyed. The reduction in number of cells is much less than in hemoglobin. (From Haden: Principles of Hematology, Lea & Febiger. Modified from Haden: J. Lab. & Clin. Med.)

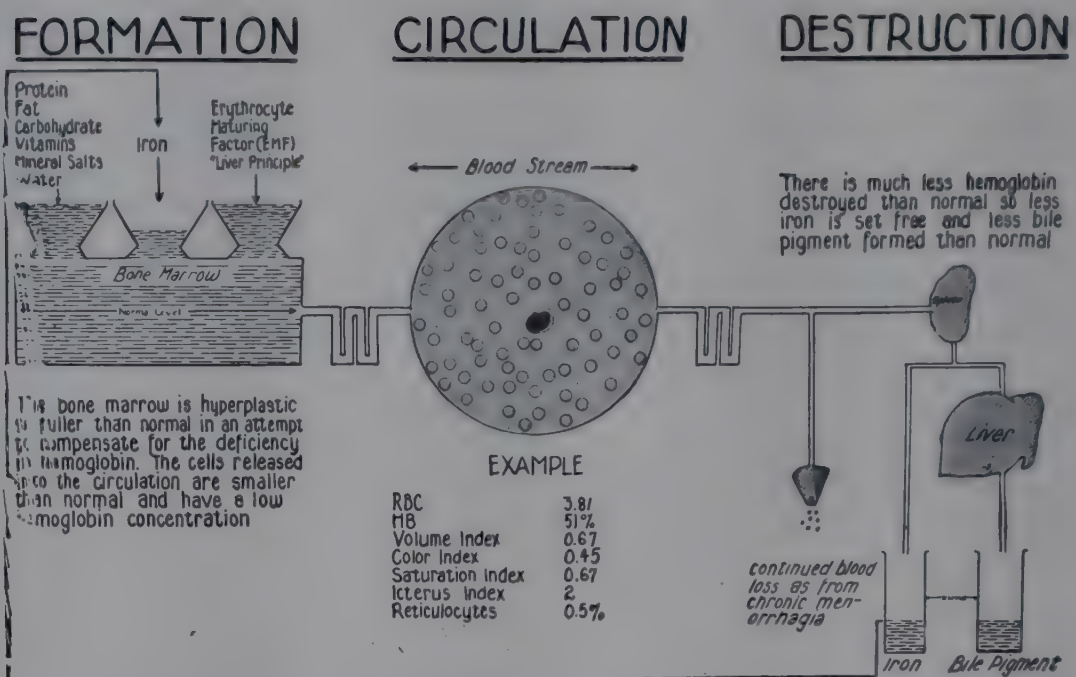


Fig. 188.—The red cells with chronic bleeding. Here there is a chronic iron deficiency so the picture is exactly like that with a deficient intake of iron (Fig. 187). (From Haden: Principles of Hematology, Lea & Febiger. Modified from Haden: J. Lab. & Clin. Med.)

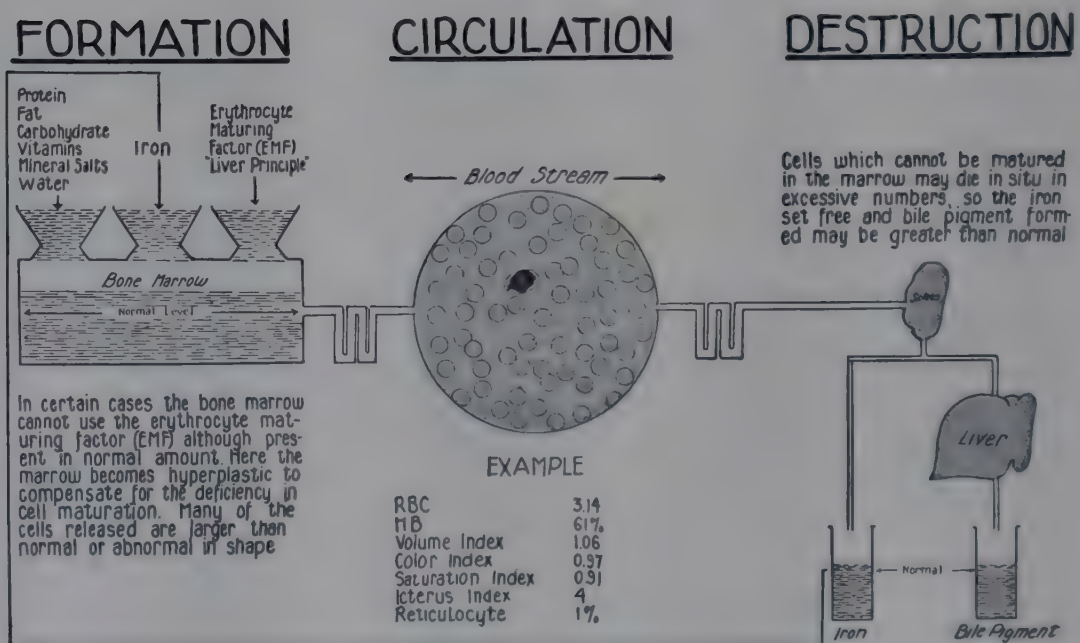


Fig. 189.—The red cells in a qualitative depression of the marrow in which the erythrocyte-maturing factor is not normally used. Here the marrow receives the specific factor but cannot use it. The picture is similar to that with an idiopathic defect in formation of the necessary factor (Fig. 186). (From Haden: Principles of Hematology, Lea & Febiger. Modified from Haden: J. Lab. & Clin. Med.)

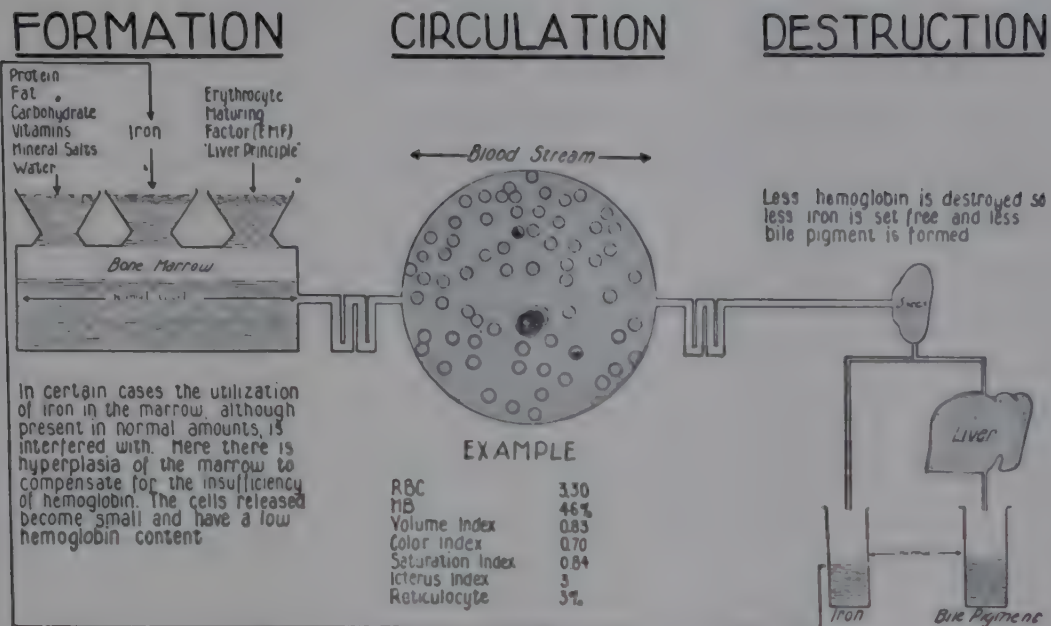


Fig. 190.—The red cells in a qualitative depression of the marrow in which iron cannot be used. Here the marrow receives iron but cannot incorporate it normally so the picture is similar to that due to an insufficient supply of iron (Figs. 187 and 188). (From Haden: Principles of Hematology, Lea & Febiger. Modified from Haden: J. Lab. & Clin. Med.)

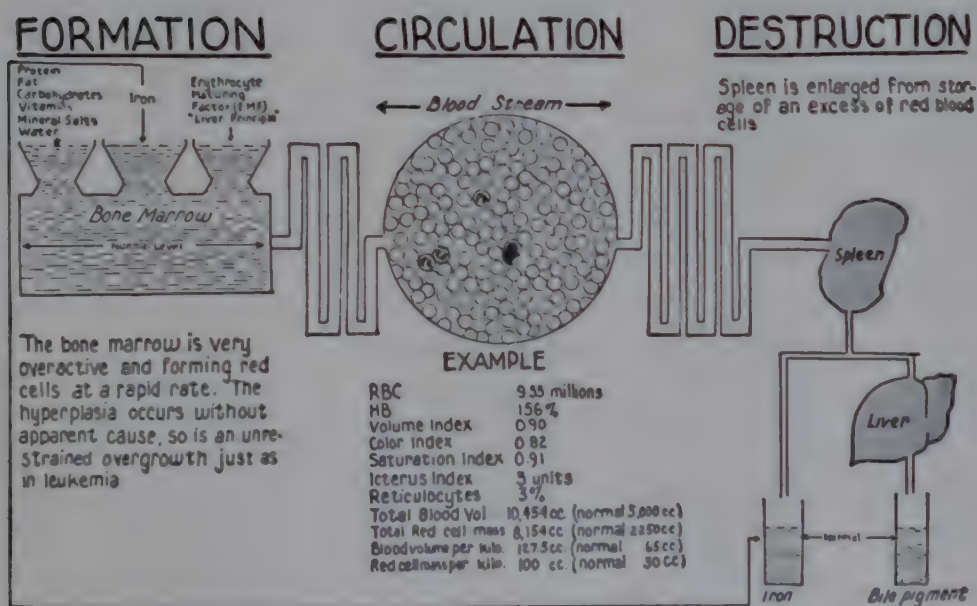


Fig. 191.—The red cells in polycythemia vera. In this disease there is an enormous overproduction of erythrocytes so the marrow is filled with erythrogenic tissue. The rate of destruction does not keep pace with red cell formation and the blood volume increases. The increase in red cells per unit volume and in blood volume gives a great increase in red cell mass. (From Haden: Principles of Hematology, Lea & Febiger. Modified from Haden: J. Lab. & Clin. Med.)

Hemorrhagic and Posthemorrhagic Anemia

Immediately following the hemorrhage there is a drop in the red count and the hemoglobin. Certain changes in the blood picture are of importance in estimating the extent of the hemorrhage and the amount of attempted regeneration on the part of the erythropoietic and leukopoietic systems.

A rapidly rising erythrocyte count, that is, a constant rise if counts are taken hourly or periodically during the day, denotes a possible hemoconcentration leading to shock.

Leukocytosis with neutrophilia and no shift is seen following hemorrhages. In severe hemorrhage there is a slight left shift. In exhausting hemorrhages there is a regenerative shift.

Marked polychromasia in the blood film (rise in reticulocytes) indicates regeneration of the erythrocytes and is a good sign following a hemorrhage. Basophilic punctation means degenerative effects, possibly toxic, on the erythrocytes. An occasional normoblast can be found in the blood in severe hemorrhages as a sign of rapid regeneration of the erythrocytes, but if many normoblasts appear, there is overstimulation of the erythropoietic system and this is regarded unfavorably. Megaloblasts are serious findings.

Blood platelets rise rapidly following a hemorrhage as a sign of regeneration of the erythrocytes. Schilling regarded this rise as proof of his theory that blood platelets represent altered forms of the nuclei of erythrocytes which are extruded as the erythrocytes leave the bone marrow. The rise in blood platelets, according to Schilling, corresponds mathematically to the number of newly formed erythrocytes which enter the blood stream following a hemorrhage.

The erythrocyte count and hemoglobin figures are used as gauges in determining whether or not transfusions are indicated, and the amount of blood to be administered.

The erythrocytes often belong to the classification microcytic hypochromic or normocytic normochromic.

Chlorosis

Chlorosis is a very interesting disease frequently seen in young women, although Castle and Minot¹ state: "At present it is very rarely observed at least in the form described in the medical literature of the preceding three centuries. Undoubtedly cases of hypochromic anemia in young women, recognized today as associated with defective diets, decreased gastric secretions, and chronic blood loss, would have been called chlorosis a generation ago." They exhibit symptoms of weakness. These patients will complain in the morning after a good night's sleep of not being refreshed by sleep, but in the afternoon and toward the evening, this weakened condition disappears. They are not necessarily pale. They complain of a tired feeling, headache, difficulty in breathing after bodily exercise, palpitation of the heart, pain in the side, and disturbances of menstruation. Dyspeptic symptoms are frequent.

¹Castle, William B., and Minot, George R.: *Pathological Physiology and Clinical Description of the Anemias*, New York, 1936, Oxford University Press.

They are often pale, but not necessarily so. The lips and visible mucosae are pale but the cheeks are not. The skin color may be pale yellow or pale green.

Clinical symptoms are as described above, plus dark spots floating before the eyes, disturbances in accommodation, and quick fatigue after reading. The retina is pale and is characterized by transparency of the blood vessels. There may be spasms with cold hands and feet. Thromboses have been described in this disease. The pulse is full, soft, and dicrotic. Blood pressure is normal. The spleen is not necessarily enlarged, according to Schilling, although von Noorden states that it is always enlarged.

One may distinguish between *puberty* chlorosis and *late* chlorosis. The first type is typical and classical; the latter type is not so typical.

Torpid chlorosis is a type of a chronic nature without any signs of regeneration in the blood.

Blood Picture in Chlorosis.—The blood picture is essentially a microcytic hypochromic anemia with a marked reduction of the hemoglobin. (See Plate XXIV.) In contrast to the hypochromic anemia of older women, gastric hyperacidity is common and achlorhydria is rare. Before a remission, the blood shows normal red blood cell content, the red cells are small and pale, and the color index is lowered. A moderate case shows hemoglobin values down to 50 or 60 per cent but the red cell count runs about normal, or about 4,000,000. This brings about a lowering of the color index. In severe chlorosis, the hemoglobin goes down to 20 per cent and the red cell count to 1,500,000 or 2,000,000. The color index and volume index are very low and the cells are very pale and small.

The most characteristic point about the blood picture of chlorosis is the presence of small and pale red cells (microcytes) and the constant lowering of the color index. With improvement, the color index is elevated.

Leukocytes in chlorosis show a normal or subnormal value, but at times there are leukocytoses up to 10,000 to 13,000. There is a lymphocytic decline in all these cases due to hypofunction of lymphatic apparatus. This lymphopenia is very characteristic.

There is a decline in the albumin content of the blood serum in all cases. They show a very pale watery serum. The diagnosis of chlorosis is largely based upon the anamnesis showing disturbances at the time of puberty in the female. There is a history of weakness and the other symptoms catalogued, which may spontaneously improve. Recurrences occur in the spring and fall, apparently without any known cause. Symptoms have already been described. These patients are usually frail, weak girls. Blood picture is an anemia of the secondary type with small pale red cells, lowered color index, and lowered red cell volume, no increase in the blood platelets; the serum is pale and hydremic.

In all probability, chlorosis was a form of hypochromic microcytic anemia. Moore² has written an interesting obituary to the so-called "idiopathic hypochromic anemias" which he believes likely are due to chronic blood loss. These cases remain idiopathic only because the site of blood loss often is not determined.

²Moore, C. V.: *Blood* 5: 876, 1950.

Severe iron deficiency anemia may occur in infants who are fed on an exclusive milk diet. We have observed such a child, aged 16 months, in whom the red count dropped to 1,600,000 and the hemoglobin was less than 5 gm. per cent. Treatment with iron cleared up the anemia.

Macrocytic Anemias

There are two factors which must be considered in the discussion of pernicious anemia and related macrocytic anemias, namely, the intrinsic and the extrinsic antianemia factors. The intrinsic factor is an indefinite something which is neither hydrochloric acid, rennin, pepsin, nor lipase. It is deficient in pernicious anemia. It is found in the gastric secretion in other types of anemia, including aplastic anemia and chronic hypochromic anemia. The extrinsic factor, which is measured by the production of a reticulocyte response in cases of pernicious anemia in relapse following its administration together with normal gastric secretion, is present in beef muscle and in many other foods. It is not ether soluble and it passes through an ultrafilter. It is known that the intrinsic factor is lacking in pernicious anemia. Macrocytic anemia occurs by reason of the lack of extrinsic factor in the diet or through a combination of dietary deficiency, insufficient intrinsic factor, or faulty absorption. Macrocytic anemia may also be caused by increased requirements, imperfect utilization, or faulty storage, or metabolism of an active hematopoietic principle in the liver.

Macrocytic anemias are classified as (1) tropical nutritional anemia, due to deficient diet; (2) following total gastrectomy, due to lack of intrinsic factor; (3) in sprue and pellagra, due to poor absorption as well as defective diet; (4) also found following resection of the intestine; and (5) in pernicious anemia of pregnancy. When macrocytic anemia is found in chronic and extensive liver disease, it is due to a faulty storage of liver principle and deranged function of the liver. The last word has not yet been written about the exact mechanism of macrocytic anemia.

Pernicious Anemia

Pernicious anemia is known also as Biermer's disease, or more properly, *morbus hunter-addisonii*. It is clinically a symptom complex of a severe megalocytic anemic type accompanied by marked intestinal symptoms, glossitis, achylia, dyspepsia and nervous symptoms, paresthesias, some cases showing the characteristic myelosis of Henneberg and various psychic changes in the individual.

This disease was formerly always considered fatal: once the diagnosis was made, the death warrant was practically signed; but with the discovery of liver therapy by Whipple, Minot, and Murphy and the therapy of hogs' stomach by Castle, this disease has largely lost its terrors; its victims have been practically recalled from death to life.

Etiology.—Pernicious anemia, as well as related macrocytic anemias, has been classified by Castle and Minot¹ as insufficient blood production due to nutritional deficiency of the bone marrow. Every type of macrocytic anemia

¹Castle, Wm. B., and Minot, George R.: *Pathological Physiology and Clinical Description of the Anemias*, New York, 1936, Oxford University Press.

showed prompt response to the administration of extracts of liver. If this liver therapy is discontinued, relapses occur. This seems to eliminate the former theories of infections, toxins, and noxae, to explain the etiology of anemia of the macrocytic type. It was discovered in 1926 by Minot and Murphy² that liver therapy was effective in the treatment of pernicious anemia. This seemed to emphasize the deficiency theory of pernicious anemia. Minot, Murphy, and Stetson³ proved that the reticulocyte response to the feeding of liver was a proof of the effectiveness of the agent. Castle⁴ and others showed that untreated patients with pernicious anemia were deficient in gastric secretion of an essential thermolabile constituent, or intrinsic factor. It was shown by Castle and Townsend⁵ that the gastric secretion of the normal individual was ineffective by itself. Similarly, beef muscle was also ineffective on administration to suitable patients with pernicious anemia. The beef muscle, called the extrinsic factor, when combined with the normal gastric secretion, increased blood production and clinical improvement similar to that induced by liver therapy.

Pernicious anemia, therefore, develops in individuals whose gastric juice contains insufficient amounts or none of the intrinsic factor to react effectively with the food. Expressed in other terms, normal gastric juice with food produces the active principle which is stored in the liver, kidney, and other tissues of normal individuals and permits normal bone marrow function to go on and thereby prevents anemia. Castle and his associates believe that pernicious anemia develops from a lack of the intrinsic or extrinsic factor or a little of each. They also believe that defects of intestinal absorption, even with a normal gastric mechanism, may lead to pernicious anemia. Sturgis and Isaacs⁶ showed that pig stomach was effective in the treatment of pernicious anemia and, therefore, this organ had the same substance present as normal gastric juice of man. The liver substance or factor that helps the bone marrow to return to normal in cases of pernicious anemia is able to resist boiling, while that in normal human gastric juice and in pig stomach is rapidly destroyed by temperatures above 70° C. Meulengracht⁷ proved that the factor is present in the pyloric region of the pig stomach.

The isolation of vitamin B₁₂ and related cobaltamines appears to have solved the problem of extrinsic factor, with which it has come to be identified. Jukes⁸ has summarized the biochemistry of folic acid and vitamin B₁₂. Vitamin B₁₂ has a molecular weight of 1550 to 1750 and contains cobalt which accounts for its red color. It is effective in microscopic quantities in producing a remission in patients with pernicious anemia when given parenterally. Oral administration is ineffective unless administered simultaneously with or shortly after ingestion of intrinsic factor. Rubbo⁹ presents the present-day concept of the enzyme systems involved in red blood cell production. He visualizes the action of folic (folinic) acid as catalyzing the pyrimidines from

²Minot, G. R., and Murphy, W. P.: *J. A. M. A.* **87**: 470, 1926.

³Minot, G. R., Murphy, W. P., and Stetson, R. P.: *Am. J. M. Sc.* **175**: 581, 1928.

⁴Castle, W. B.: *Am. J. M. Sc.* **178**: 748, 129.

⁵Castle, W. B., and Townsend, W. C.: *Am. J. M. Sc.* **180**: 305, 1930.

⁶Sturgis, C. C., and Isaacs, R.: *J. A. M. A.* **97**: 747, 1929.

⁷Meulengracht, E.: *Acta med. scandinav.* **82**: 352, 1934.

⁸Jukes, T. H.: *Fed. Proc.* **12**: 633, 1953.

⁹Rubbo, S. D.: *M. J. Australia* **1**: 324, 1953.

methionine. The second stage involves vitamin B₁₂ as the catalyst for a combination of the pyrimidines and ribose (or desoxyribose) to form thymidine (nucleosides). Although a considerable amount of work has been done on vitamin B₁₂, showing its relation to nucleoprotein synthesis, there still remain many gaps in our knowledge of this complex mechanism. Assay of vitamin B₁₂ activity has been carried on by determination of growths of *Lactobacillus leishmanii*, *Escherichia coli* mutants, *Euglena gracilis*, and *Pateriochromonas stipitata*.

Glass and associates,¹⁰ using *Euglena gracilis* for assay, found oral administration of vitamin B₁₂ together with a potent intrinsic factor concentrate caused a sharp rise in vitamin B₁₂ blood level, but less than 0.5 per cent in the urine. A rise in urinary output of vitamin B₁₂ occurred after the reticulocyte peak.

Vitamin B₁₂ has been linked with various isotopes for experimental studies which have yielded a considerable store of information, most of which has been directed at determining mechanisms involved in the interaction of the various factors involved in macrocytic anemias. Tests based on the low urinary output of orally ingested radioactive vitamin B₁₂ have been used to detect pernicious anemia patients in remission.¹¹

The attack on the nature of intrinsic factor continues unabated and the elusiveness of this factor continues to interest and baffle investigators. Castle¹² has reviewed the development of knowledge concerning the gastric intrinsic factor and its relation to pernicious anemia. He concludes, after a scholarly exposition with extensive bibliography, that he is "sure that there is now general agreement at least on the present state of uncertainty about the nature and function of the intrinsic factor." He presents several theories, favoring the "catalyzed absorption theory" which assumes that intrinsic factor directly and specifically facilitates the absorption of vitamin B₁₂ by the intestinal wall. According to Castle, pernicious anemia "would not develop if the patient could effect daily the transfer of a millionth of a gram of vitamin B₁₂ the distance of a small fraction of a millimeter across the intestinal mucosa and into the blood stream. This he cannot do, principally as a result of failure of his stomach to secrete into its lumen some essential but unknown substance."

The symptoms of the disease are as follows: For a long period, patients show a gradually increasing fatigue with loss of appetite, vague symptoms of gastric disturbance, achylic in nature, with burning of the tongue or about the gums. These patients at this time display the so-called "Hunter tongue" or the "Moeller" glossitis, with rhagades about the mouth, gingivitis, marked dental caries, and extreme pallor. In these cases we note the light yellowing of the sclerae and other signs of possible hemolytic icterus. They show a golden yellow serum due to bilirubinemia as determined by the alcoholic (indirect) reaction of van den Bergh. We find here a dynamic icterus, urobilinuria and urobilinogenuria.

¹⁰Glass, G. B. J., Lillick, L. C., and Boyd, L. J.: *Blood* 9: 1127, 1954.

¹¹Schilling, R. F.: *J. Lab. & Clin. Med.* 42: 946, 1953.

¹²Castle, W. B.: *New England J. Med.* 249: 603, 1953.

There is usually complete achylia. The blood may or may not in the beginning show anemia, hyperchromia, and increase in size of red cells, while hemoglobin and total red cell values persistently remain normal. One must emphasize at this point that the most significant indication of pernicious anemia is the *increase in the size of the red cells*. The general impression of these patients is that there is something definitely wrong: unfortunately, unless they are thoroughly examined, they are likely to be labeled "neurasthenics," "gastric" or even "cardiac" and "liver" patients. The true diagnosis is suggested by the appearance of the tongue, the so-called Hunter tongue, or glossitis, which consists of little blistered elevations on the margins of the tongue, with some painful spotty areas together with flattening of the normal papillae. These patients now show achylic symptoms with a feeling

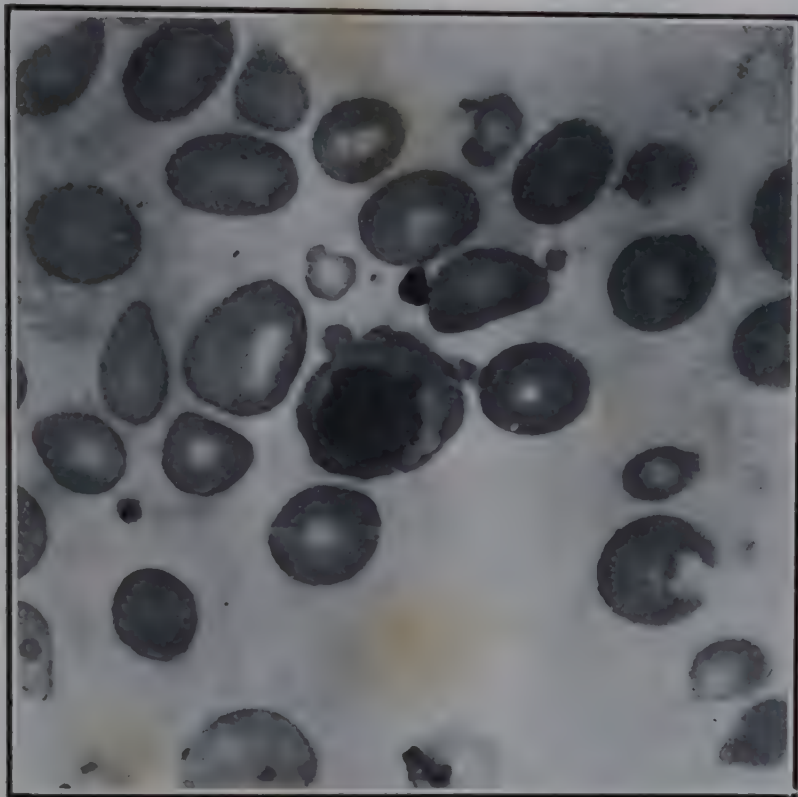


Fig. 192.—Pernicious anemia, blood film, showing hyperchromia, anisocytosis, poikilocytosis, macrocytosis, and a megaloblast. ($\times 950$.)

of fullness in the stomach or sudden diarrhea. They may develop nervous symptoms such as a numbness in the finger tips, which is due to disturbances in the terminals of sensory nerves. They also have disturbances in "taste," slight difficulty, too, with an otherwise good memory; they may show beginning impotency, perhaps slight unsteadiness in the gait; decreased or increased patellar reflexes, Babinski, paralytic or spastic ataxia.

Examination of these patients is suggestive of endocarditis, sepsis, carcinoma of the stomach, syphilis, cholangitis, chronic occult bleeding, or even a simple constitutional anemia.

There is another very remarkable phenomenon in pernicious anemia known as a "remission"—an unaccountable and sudden return to normal without any treatment. It is explained on the basis of an endocrine disturbance which at times, for some unknown reason, improves so that the patient returns to a normal status. The endocrine secretion, in other words, automatically works for the betterment of the patient and, at some time later, suddenly ceases to work, putting the patient once more into his former low status.

ADVANCED PERNICIOUS ANEMIA

BEFORE TREATMENT



1. Giant hypersegmented neutrophile
2. Monocyte
3. Lymphocyte
4. Cabot ring body and basophilic punctation in a macrocyte
5. Large blood platelet
6. Megalocyte
7. Poikilocyte
8. Polychromatic megalocyte
9. Polychromatic normocyte

Note anisocytosis, poikilocytosis, polychromasia, basophilic punctation. Erythrocytes are macrocytic hyperchromic.

AFTER LIVER THERAPY



1. Reticulocytes
2. Plasma cell
3. Polychromatic erythrocyte showing no reticulum
4. Blood platelets
5. Segmented neutrophile
6. Polychromatic microblast

ADVANCED PERNICIOUS ANEMIA

BEFORE TREATMENT



BLOOD FILM—GIEMSA STAIN

× 950

AFTER LIVER THERAPY



SUPRA-VITAL STAIN

BRILLIANT CRESYL BLUE—GIEMSA STAIN

× 950

PLATE XV.

The outcome of these cases is now favorable, but, as stated before, pernicious anemia was formerly considered a severe fatal anemia, preceded by a failing heart, edema of the lungs and subcutaneous tissues. Many of these cases developed the well-known funicular myelosis or Henneberg's myelosis; a severe spastic or tabes-like status, complicated with decubitus, bladder and intestinal paralyses. Many cases showed preagonally hemorrhagic diatheses, at times definite retinal hemorrhages. Occasionally, a patient dies with symptoms that are highly suggestive of aplastic anemia.

Blood Picture.—

The blood is watery, consequently spreads are very thin. The total white count is low, usually around 4,000 per cubic millimeter. The red cell count is low, 2,000,000 to 3,000,000 per cubic millimeter, sometimes as low as 320,000. Color index is high, owing to the disproportion between the count and the hemoglobin percentage. Color index may be 1.2, or 1.4, or even 2.0. In "remissions" the color index returns to 1.0. The blood serum has a golden yellow appearance so well described by Naegeli, with the alcoholic (indirect), van den Bergh test *positive* and the *watery* (direct) test *negative*. There is in this disease a megaloblastic blood picture with anisocytosis, poikilocytosis, and all degenerative forms of blood cells. The red cells, as stated above, are always definitely enlarged. There is no other form of anemia which shows such enlargement of red cells as we find in this disease. We find large hyperchromic cells, megalocytes, and occasionally a megaloblast. Particular attention must be called to the fact that a megaloblast as such shows a large circular nucleus with loosely woven chromatin and with a polychromatic or basophilic cytoplasm. Normoblasts may be large but they are not necessarily megaloblasts. Be careful to note the difference between a large normoblast and a megaloblast. It must be remembered, too, that *normoblasts* are often found in this disease but true *megaloblasts* are only occasionally found.

The white cells show a neutropenia and lymphocytosis with a characteristic disturbance or elevation of the monocytes; these monocytes show increased and abnormal nuclear margins. **The nuclei of the neutrophils are highly segmented.** There are sometimes 6 to 8 segments to the cell, united to each other by fine stringlike connections. Arneth called this his characteristic "right shift" or *hypersegmentation*.

Blood platelets are absolutely decreased and relatively increased and are irregular in form and size. The entire blood picture has been called the *Naegeli syndrome*.

"Blood crises" are present in the beginning of the remissions of pernicious anemia or shortly before death. Here we see many polychromatic and nucleated reds and also many regenerative leukocytes such as juveniles, myelocytes, promyelocytes, and even myeloblasts. The following hemogram is from a blood crisis during pernicious anemia.

Bas.	Eos.	Myel.	Juv.	Stab	Seg.	Lymph.	Mono.
-	-	-	2	15	68	7	8

For every 100 leukocytes there were 1,385 polychromatic normoblasts, 25 polychromatic macroblasts, 3 polychromatic megaloblasts, 2 cells in mitosis.

There were marked karyorrhexis; basophilic punctation, coarse; Howell-Jolly bodies; marked polychromasia; marked anisocytosis; and occasional myelocytes.

It is our custom to call a blood picture, in the absence of clinical symptoms, a *pernicious anemic blood picture* in reference to the above findings, but not necessarily a case of "*pernicious anemia*." It is interesting to note that from two to four days after the institution of liver therapy, there is a marked polychromasia and a marked increase in the reticulocytes. These can be well seen in the thick drop or with the supravital method. The red cells return slowly to their normal form and color. The existing hemolysis ceases. There is also a rise in the hemoglobin, together with a rise in the number of red cells, but the rise of the red cells is faster than the rise of the hemoglobin, thus making the color index fall below 1.0. Leukocytes take on the aspect of a hyperleukocytosis, often accompanied by an eosinophilia which has been described as an allergic reaction.

Diagnosis.—The Ehrlich-Naegeli syndrome, as above stated, must be found in order to think of this disease. The essential characteristic of the blood picture is a hyperchromia, marked anisocytosis, and megalocytosis. The increase in the size of the red cells is one of the most significant characteristics of pernicious anemia. There is a blood platelet change, leukopenia with lymphocytosis, a disturbance in monocytes, and a high segmentation of the neutrophils or a **right shift**. There are also oligocythemia, decreased hemoglobin and usually an elevated color index. By elimination, carcinoma, syphilis, *Diphyllobothrium latum* infestation, pregnancy, and sprue can be ruled out. One of the best methods for proving that a given case shows megalocytes, or increase in size of red cells, is to make a film from the suspected case and stain it in the ordinary manner with Giemsa. After it is dry, a very thin spread of normal blood is prepared *over this stained specimen*, allowed to dry, and then examined. The difference in size between the normal red cells and those of the given case can then be easily determined.

Hypersegmentation of the Neutrophils.—It has been known for a long time that there is a greater segmentation of the neutrophilic nucleus in pernicious anemia than in any other type of anemia. This was seen in the Arneth count when the number of cells with nuclei containing 3, 4, 5, or more segments is much higher than in normal blood. The increase in the cells of groups 4 and 5 of the Arneth count has been described by him as a "shift to the right" and is characteristic of the megaloblastic anemias. Some writers definitely state that this phenomenon is absolutely diagnostic of pernicious anemia, but it is difficult to agree with this statement in the light of the authoritative literature on the subject. In a monograph by Sturgis and Isaacs,¹ the following statement covers this question exceedingly well: "The polymorphonuclear neutrophils in pernicious anemia may have a greater percentage of nuclei with more lobes than normal; that is, the percentage of cells containing nuclei with 3, 4, 5, or more lobes is much greater in the blood of patients with pernicious anemia than in normal blood. This corresponds to what is described as a 'shift to the right' in the Arneth count, and it occurs frequently when the anemia is severe. While some regard this as diagnostic

¹Sturgis, Cyrus C., and Isaacs, Raphael: *Handbook of Hematology*, Vol. 3, p. 2251, Hal Downey, Editor, New York, 1938, Paul B. Hoeber, Inc.

of pernicious anemia, it is not characteristic of this condition alone as the same finding may be observed in other types of anemia. A 'shift to the left' takes place during the early stages of the remission."

Fleming² pointed out that the conditions in which a shift to the right occurs are pernicious anemia, sprue, and lymphocytic leukemia. Our own experience has noted its occurrence quite frequently in leukemia. Piney states that it occurs in certain cases of Hodgkin's disease prior to x-ray therapy. Davidson and Gulland³ state that the cell bodies of the polymorphonuclear leukocytes are oval and not round in this condition and that this applies both to the large forms and those of ordinary size; further, that this change is most commonly found in severe cases, and may affect all or nearly all the cells of that type to be seen in the blood film.

There are certain high lights in the diagnosis of this disease which must be emphasized: (1) Moderate anemia. (2) Changes in the blood platelets. (3) Erythrocytic changes, particularly increase in size. (4) Variations in size of the red cells. (5) Basophilic punctation, Cabot ring bodies, and polychromasia. (6) Occasional finding of megaloblasts. (7) Finding of normoblasts or nucleated red cells. (8) Neutropenia with high segmentation of the neutrophils, or Arneth's shift to the right. (9) Response of such cases to specific treatment. (10) Marked increase of reticulocytes first, after liver therapy, followed by a rise in the number of red cells before the hemoglobin begins to rise and consequent lowering of the color index is highly suggestive of the accuracy of the diagnosis of morbus addisonii. (11) Macrocytosis should attract one's attention to this disease even before an anemia is noted. (12) A high or even a normal white count almost rules out this disease in cases of suspected anemia. In all cases one finds a lowered white blood count, sometimes down to 4,000 per cubic millimeter. (13) It is claimed by Rous and Robertson⁴ that the small red cells or microcytes with pointed tails, which may be called poikilocytes, are indicative of an increased blood destruction. (14) Many basophilic punctated red cells are seen in this disease just as they are found in lead poisoning, but as a rule, *polychromatic young red cells* are not found in Addison's anemia except at the onset of improvement of a treated case. It is for this reason that the count of the reticulocytes is very important. (15) There are very low red blood counts in this disease, sometimes as low as 500,000. Carr⁵ reported 6 out of 148 cases with counts below 500,000. The lowest count in Carr's series was 320,000. Quinke reported a case with a count of 320,000. (16) The white count is very low due to the failure of the bone marrow to produce neutrophils. In other words, the lymphocytes are not interfered with, while the neutrophils are lowered. Consequently, there is a picture of relative lymphocytosis. The higher the lymphocytosis, the severer the prognosis, for the reason that the higher the lymphocytes, the more obvious it is that the bone marrow function for the manufacture of the neutrophils is partially destroyed. (17) As to the size

²Fleming, G. W. T. H.: Brit. M. J. 1: 638, 1929.

³Davidson, L. S. P., and Gulland, G. L.: Pernicious Anemia, p. 159, St. Louis, 1930, The C. V. Mosby Company.

⁴Rous and Robertson: J. Exper. Med. 25: 651 and 665, 1917.

⁵Carr, J. G.: Am. J. M. Sc. 160: 737, 1920.

of the red cells, the majority exceed 8.5 microns in diameter. (18) The fragility test in this disease: the red cells in hypotonic salt solutions are normal, while in hemolytic jaundice their resistance is lowered.

Reticulocyte Response After Erythrocyte Maturation Factor Therapy.—

One of the most important phenomena in connection with the prognosis of this disease is the reticulocyte response after liver or other erythrocyte maturation factor therapy. In other words, after the use of these standard remedies, reticulocytes increase markedly in the peripheral blood. This is due to the increased maturation of the nucleated red blood cells in the bone marrow. This reticulocyte response does not show in the first five days of treatment for the reason that in this period the megaloblasts are developing into reticulocytes in the bone marrow. After the fifth day, there is a rapid increase in the number and percentage of reticulocytes. After the ninth to tenth day, there is a decrease in the percentage of reticulocytes accompanied by an increase in the total number of red blood cells. From time to time, thereafter, there are increases in the percentage of reticulocytes due to the stimulation of blood formation. The greater the dose of the remedial agent used, the more rapid will be the reticulocyte response. This is dependent, too, upon the route of administration, whether by mouth or by injection. The depth of the anemia in part determines the height of reticulocytosis; the more anemic the patient, the higher the reticulocyte response.

Folic Acid Therapy.—Some patients show a sensitivity to liver: inflammation around the needle point after injection, together with generalized itching sensitization of the skin. In such cases, recourse must be had to the use of folic acid (see pages 725, 1379, 1380). This is a vitamin which is known at this time to be very useful in the treatment of general macrocytic anemias, particularly the nutritional anemia in the southern part of the United States and the tropics. It is highly useful in sprue, in pernicious anemia of pregnancy, in anemias associated with chronic alcoholism, and in carcinoma of the stomach.

It has been well established that folic acid will cause a complete hematologic response and will maintain blood levels in patients with pernicious anemia. However, the neurological manifestations of this disease may continue to progress with folic acid therapy even though the hematologic response is satisfactory. For this reason, the use of folic acid *alone* is not recommended for the treatment of pernicious anemia.

Vitamin B₁₂ Therapy.—

In 1948 Smith¹ and Rickes and co-workers² isolated crystalline vitamin B₁₂ from liver. It was found that infinitesimally small amounts of this substance were effective in restoring normal hematologic values in pernicious anemia patients in relapse. The dosage schedule is a minimum of 1 µg per day, but larger dosages are generally used. Blackburn³ and Piney⁴ have re-

¹Smith, E. L.: *Nature* **161**: 638, 1948.

²Rickes, E. L., Brink, M. G., Koniuszy, F. R., Wood, T. R., and Folkers, K.: *Science* **107**: 396, 1948.

³Blackburn, E. K., *Med. Illus.* **7**: 511, 1953.

⁴Piney, A.: *Med. Illus.* **7**: 556, 1953.

viewed the modern treatment of pernicious anemia in excellent fashion. Our schedule has been 50 μ g intramuscularly daily for 7 to 10 days until a maximal reticulocyte response is obtained. Thereafter, 50 μ g dosage every second day is maintained until the erythrocyte and hemoglobin values are restored to almost normal values. Maintenance dosage of 20 to 30 μ g every two weeks appears quite adequate to maintain normal hematologic and neurological balance. Single doses greater than 50 μ g do not add to the effectiveness of treatment because amounts above a threshold value of 25 to 50 μ g by injection are rapidly and quantitatively excreted in the urine.⁵ Single massive doses at infrequent intervals cannot be substituted for regular injections at fairly frequent periods (two to three weeks) without the danger of relapse and the aggravation of central nervous system disease.⁵

A great deal of attention has recently been centered on the use of vitamin B₁₂ orally, especially when combined with mucosal extracts of hog stomach.⁶ Small doses of vitamin B₁₂ below 20 μ g are ineffectual when given alone, but when combined with intrinsic factor containing mucinous materials from hog stomach, responses are optimal or suboptimal. These observations are important in elaborating our basic understanding of the factors involved in pernicious anemia, but hematologists generally are opposed to oral preparations indiscriminately used. Suboptimal dosages may mask symptoms until irreversible neurological changes take place. The use of minimal dosages is decried because such dosages alter the bone marrow picture sufficiently to make diagnosis impossible and to date we have no clinical method of making an accurate diagnosis of pernicious anemia that supersedes the bone marrow picture in relapse.

Schilling⁷ has described a new test for intrinsic factor in which he has attempted to solve the dilemma of diagnosis in treated patients with pernicious anemia. The test consists of the administration of radioactive vitamin B₁₂ (B₁₂ Co⁶⁰) orally, followed in 2 hours by 1,000 μ g of nonradioactive B₁₂ parenterally. In normal subjects radioactive vitamin B₁₂ appears in the urine within 4 to 6 hours after the oral dose and this excretion continues for 24 hours. In pernicious anemia, no radioactive substance appears in the urine.

Another approach to diagnosis in the treated patient with pernicious anemia is the study of exfoliated material obtained by gastric lavage. Rubin and Massey⁸ have demonstrated a cell type in such washings which appears specific for pernicious anemia, and which was found in 20 of 21 patients who had been under therapy for years. These cells are markedly enlarged atypical columnar epithelial cells with nuclei approaching twice normal size.

Olmstead and Hirschboeck⁹ studied the fasting urorennin excretion in 50 patients with pernicious anemia and found that none of these patients excreted more than 0.5 rennin unit per 10 c.c. of urine. In a control group aged 40 to 90 years, 13 excreted less than 0.5 rennin unit, but the others excreted more than this. Their conclusions are that if the morning urorennin

⁵Reisner, E. H., and Weiner, L.: *Blood* 8: 81, 1953.

⁶Glass, G. B. J., and Boyd, L. J.: *Blood* 8: 867, 1953.

⁷Schilling, R. F.: *J. Lab. & Clin. Med.* 42: 860, 1953; *ibid.*, p. 946.

⁸Rubin, C. E., and Massey, B. W.: *J. Lab. & Clin. Med.* 42: 942, 1953.

⁹Olmstead E. G., and Hirschboeck, J. S.: *Am. J. M. Sc.* 226: 84, 1953.

excretion exceeds 0.5 rennin unit, it is likely that pernicious anemia is quite remote. On the other hand, excretions below 0.5 unit only imply the possibility of pernicious anemia but do not make a diagnosis.

The relationship of vitamin B₁₂ to the extrinsic and intrinsic factors of Castle continues to be of considerable interest; the subject has been exhaustively reviewed by Castle.¹⁰ He and his co-workers¹¹ believe that they have confirmed his theory that the primary function of intrinsic factor is to enhance the assimilation of vitamin B₁₂. Although many investigations are current, problems related to this phase of pernicious anemia are provocative.

Bone Marrow in Pernicious Anemia.—The principal change in the bone marrow in pernicious anemia is a replacement of the fatty marrow, normally found in certain bones, with a soft red marrow normally found in the sternum and ribs. There is an active increase of megaloblasts. Davidson and Gulland¹² found that the number of white cells in the bone marrow is greater than might be expected from the constant leukopenia present in the peripheral blood in these cases. Davidson and Gulland found also that the segmented neutrophils were reduced in number in the bone marrow and that the predominant type of leukocyte present was either the myelocyte or the lymphocyte. There is a marked decrease in the number of megakaryocytes. With improvement of the patient the large number of megaloblasts is changed into a condition where we see a greater number of normoblasts and erythrocytes. Doan and Zerfas¹³ found in supravital studies of the bone marrow in five cases of pernicious anemia 0.5 to 8.1 per cent polymorphonuclear neutrophils; 12.8 to 62.9 per cent neutrophilic myelocytes; 1.6 to 30.3 per cent eosinophilic myelocytes; 0.1 to 3.6 per cent basophilic myelocytes; 0.3 to 19.7 per cent myeloblasts; 0.0 to 0.8 per cent primitive cells; 3.4 to 6.7 per cent clasmocytes; 4.3 to 13.1 per cent monocytes; 0.0 to 0.6 per cent megakaryocytes; 5.8 to 20.1 per cent lymphocytes; no lymphoblasts; 0.0 to 1.5 per cent eosinophiles; 0.0 to 0.2 per cent basophiles; 0.6 to 14.4 per cent megaloblasts; 1.0 to 3.2 per cent normoblasts; 0.5 to 1.6 per cent unclassified.

The mechanism of the bone marrow changes is explained very well by the work of Minot and Murphy¹⁴ who showed that the red cells fail to mature because of the absence of a specific product of gastric digestion. When sufficient amount of this substance, which is supplied by erythrocyte maturation factor, is present in the blood, cells mature properly, and when this material is deficient, the development of the cells stops at the megaloblastic point, and a relapse occurs.

For further description of bone marrow in pernicious anemia, refer to page 932.

Sprue*

Sprue has been described as an insidious, chronic, remittent catarrhal inflammation of the alimentary canal occurring particularly in Europeans

*This article has been contributed through the courtesy of Dr. Ramón M. Suárez, of the Hospital Mimiya, Santurce, Puerto Rico. The tables and the microphotograph of the bone marrow were also furnished by Dr. Suárez. Dr. Suárez has had an unusual opportunity to study this disease in Puerto Rico.

¹⁰Castle, W. B.: *New England J. Med.* 249: 603, 1953.

¹¹Wallerstein, R. O., Harris, J. W., Schilling, R. F., and Castle, W. B.: *J. Lab. & Clin. Med.* 41: 363, 1953.

¹²Davidson, L. S. P., and Gulland, G. L.: *Pernicious Anemia*, St. Louis, 1930, The C. V. Mosby Co.

¹³Doan, C. A., and Zerfas, L. G.: *J. Exper. Med.* 46: 511, 1927.

¹⁴Minot, G. R., and Murphy, W. P.: *J. A. M. A.* 87: 420, 1926.

who are residing or have resided in tropical or subtropical climates. Undoubted cases of sprue have been reported during recent years in people living in temperate climates, who had not visited the tropics. These cases appear in the literature as "nontropical sprue."

The disease is characterized by stomatitis and a bare, raw, painful tongue, recurrent attacks of cramps and diarrhea, voluminous, frothy, acid stools, loss of weight, and a severe anemia of the pernicious type.

The onset is usually insidious, and manifests itself first by soreness of the buccal mucosa, and vague digestive disturbances. The course is afebrile. The untreated patient gradually becomes weak, emaciated, irritable, and deadly pale.

The etiology is unknown. Only the concept of a deficiency disease has been able to withstand prolonged investigation. Castle, Rhoads, and their collaborators, while working in Puerto Rico, confirmed the belief that deficient diets frequently antedate the onset of clinical sprue. Surely the disease is not contagious. The theory of the "monilia psilosis" as a causative agent has been discarded.

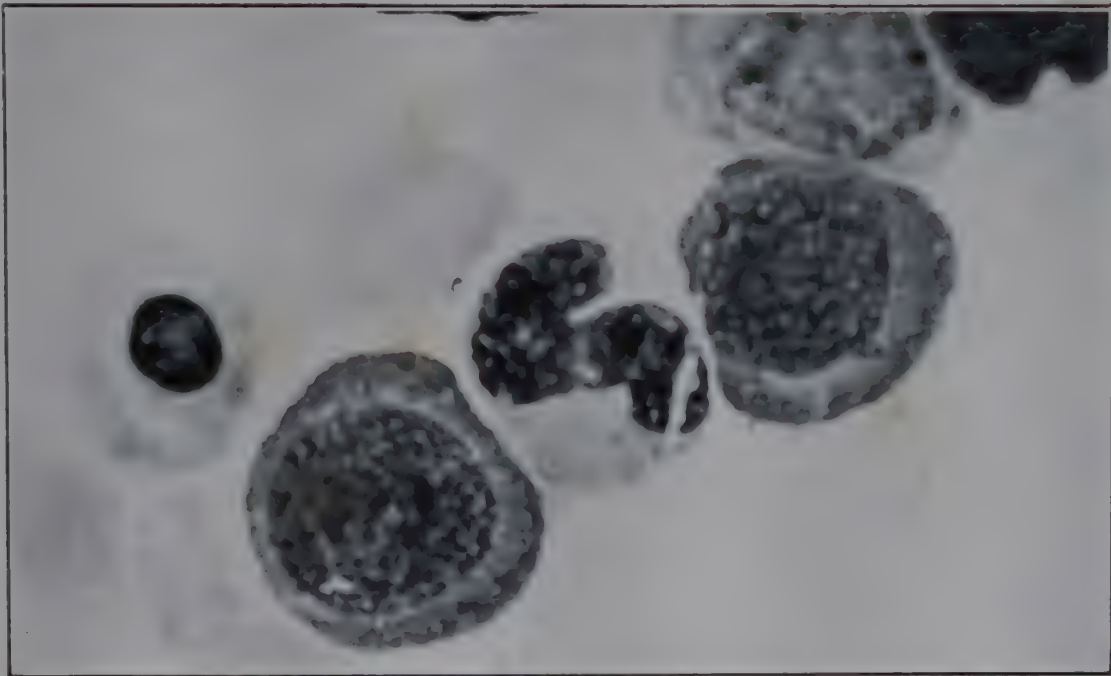


Fig. 193.—Bone marrow showing megaloblasts, a large normoblast, and a neutrophilic juvenile cell. (Courtesy Dr. Ramón M. Suárez, Puerto Rico.)

The disease is endemic in the East Indies, and in South China, in India and Ceylon, in the West Indies and in Australia, in Japan, Korea, and the Philippine Islands. Its occasional occurrence in the United States is an accepted fact.

In a series of 150 cases studied in Puerto Rico, there were 121 whites, 26 mulattoes, and only 3 Negroes. The disease appears to be, therefore, extremely rare in the colored race. One-third of the patients were less than 40 years old, a point of difference from true Addisonian anemia.

In general appearance some of the older patients presented strong resemblance to persons with pernicious anemia. Loss of subcutaneous fat, and wrinkling and pigmentation of the skin, especially of the arms and legs, favor the diagnosis of sprue. In contrast to pernicious anemia the spleen was always normal or small. Although purpuric or scorbutic spots were occasionally found, retinal hemorrhages were uniformly absent.

The occurrence of osteoporosis and osteomalasia, reported as a common finding in nontropical sprue, was not found in the series of 150 cases already mentioned.

Investigations of the glucose tolerance showed uniformly a flat curve, favoring the theory of deficient intestinal absorption, and making the hypothesis of a possible pancreatic disease less important in the understanding of sprue. Moreover the pancreas has been found normal in a high percentage of cases studied at autopsy.

The anemia in sprue is macrocytic, usually hyperchromic, and rarely hypochromic. The mean cell volume averages 123.6 cubic microns, the color index 1.22. Nucleated red cells are very rarely found. Eosinophiles are reduced. The white cell count is below normal, averaging 5,280.

SPRUE 150 CASES	LOWEST	HIGHEST	AVERAGES
Hemoglobin	2.4 gm. (16.5%)	14.8 gm. (102%)	9.7 gm. (66%)
Erythrocytes	690,000	4,410,000	2,710,000
Packed Cells	10.5 mm.	44 mm.	33.49 mm.
Leukocytes	1,550	13,600	5,280
Color Index	0.64	2.2	1.22
Volume Index	1.0	2.3	1.39
Mean Cell Volume	89 c. microns	220 c. microns	123.6 c. microns
Mean Cell Hgb.	26 m. gm.	59 m. gm.	36.6 m. gm.
Mean Cell Hgb. (conc.)	20%	45%	26.1%

Studies of the aspirated sternal marrow yield interesting data. Megaloblasts are frequently seen. Usually there are more erythroblasts than normoblasts. Megakaryocytes, plasma cells, and monocytes were very rarely seen.

The second table shows the differential counts of the nucleated elements in the aspirated sternal marrow of sprue cases before treatment, and again 10 days and 2 months following the institution of parenteral liver therapy. The erythroblastic elements, originally 41.73 per cent, gradually diminished to 15.83 per cent; the myeloblastic cells increased from 53.5 per cent to 78.79 per cent; and the lymphoblastic elements in the marrow remained stationary during the observation period of 2 months.

The van den Bergh test is the same in sprue as it is in pernicious anemia; i.e., the test points to an increased degree of blood destruction. There is ap-

BONE MARROW IN SPRUE
(AVERAGES)

	INITIAL	10 DAYS	2 MONTHS
Megaloblasts	6.31	1.79	0.60
Erythroblasts (Early)	8.71	3.06	1.17
Erythroblasts (Late)	11.12	5.23	2.80
Normoblasts	15.59	11.68	11.26
Myeloblasts	0.72	1.24	0.63
Promyelocytes	2.60	3.53	3.40
Myelocytes (Neut.)	17.83	16.46	17.51
Myelocytes (Eos.)	2.77	3.54	2.51
Myelocytes (Baso.)	0.30	1.78	0.40
Polynuclears (Neut.)	26.92	44.14	51.91
Polynuclears (Eos.)	1.91	1.26	2.43
Monocytes	0.03	0	0
Lymphocytes	5.11	5.95	4.89
Megakaryocytes	0.07	0.24	0.06

proximately normal or slightly increased resistance of red cells to hemolysis by hypotonic sodium chloride solution, which is just about what we find in pernicious anemia. A reduction in blood platelets may occur, but it is variable and not to be depended upon as a diagnostic measure.

The most striking pathologic finding at autopsy is the almost total disappearance of the subcutaneous fat. The intestines are markedly thinned out, this atrophy especially affecting the mucosa. All internal organs are smaller, especially the liver and heart. Histopathologic changes of a chronic or ulcerative colitis were found in 30 per cent of the cases.

Clinically, hematologically, and pathologically many of the cases of sprue are indistinguishable from Addisonian anemia. Low blood calcium figures have been reported in sprue, which is not the case in pernicious anemia. Achlorhydria, an almost constant finding in pernicious anemia, is found in only 30 per cent of sprue cases. Young children and adolescents are frequently affected with tropical sprue, and very rarely, if ever, with pernicious anemia. Cord changes or neurological manifestations, on the other hand, are extremely rare (about 2 per cent) in sprue.

The two most important factors in the treatment of sprue are an appropriate diet and adequate liver or folic acid therapy. Ashford* insisted on the necessity of a high protein, low fat and low carbohydrate diet. Liver extract should be administered parenterally. Both the diluted and the concentrated liver extracts have been found highly effective in all cases of sprue characterized by a macrocytic anemia, whether hyperchromic or hypochromic. Sprue is more resistant to treatment than pernicious anemia, and requires larger doses of liver. The use of folic acid in the treatment of sprue is discussed on pages 720, 1379, 1380.

Hemolytic Anemia

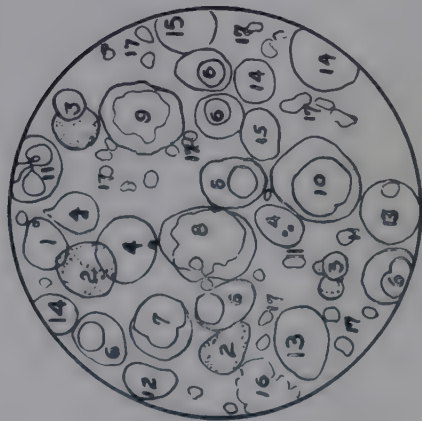
There are a number of blood dyscrasias which are grouped together as due to increased blood destruction. Paroxysmal hemoglobinuria, familial and acquired hemolytic jaundice, and sickle cell anemia are due to increased susceptibility of the red blood cells to destruction. There is a hemolysin present in the plasma in paroxysmal hemoglobinuria. In sickle cell anemia and hemolytic jaundice, where there is a physical peculiarity of the erythrocytes, precise knowledge of its relation to increased blood destruction is lacking, although recent advances in the knowledge of "molecular disease of hemoglobin" has given us a new lead as to the basic disturbances.

All cases of hemolytic anemia have in common the symptom of blood destruction.

Hemolytic anemias may be divided into acute, subacute, and chronic types. The acute cases are due to malaria and other protozoa; Bartonellae; bacterial toxins; chemical agents such as phenylhydrazine, saponin, trinitrotoluene, dinitrobenzol, aniline, amido and nitro compounds of phenol, benzol, toluol, lead, etc.; snake venoms; extensive burns; mismatched transfusions of isoagglutinogens A and B. All other types are those due to intragroup

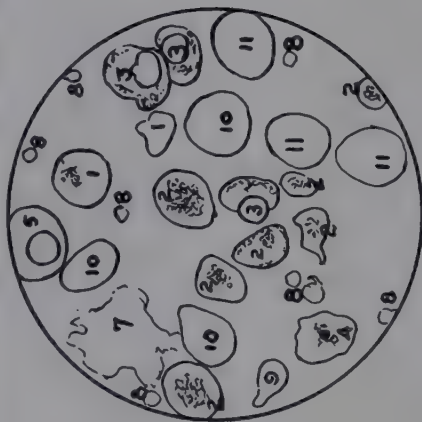
*Ashford, B. K.: J. A. M. A. 91: 242, 1928.

IDIOPATHIC HEMOLYTIC ANEMIA



BLOOD FILM
GIEMSA STAIN $\times 950$

1. Marginal granules
2. Basophilic punctation
3. Normoblast with basophilic punctation
4. Howell-Jolly bodies
5. Normoblast with Howell-Jolly body
6. Normoblast
7. Lymphocyte
8. Monocyte
9. Basophilic myelocyte
10. Neutrophilic myelocyte
11. Segmented neutrophile
12. Erythrocyte showing karyogenic metachromasia
13. Endoglobular degeneration
14. Target cells (1)
15. Polychromatic erythrocyte
16. Fragmentary nuclear structure
17. Blood platelets. Erythrocytes show anisocytosis, poikilocytosis, polychromasia, basophilic punctation, and some hypochromia



BLOOD FILM—RETICULOCYTES
SUPRAVITAL STAIN $\times 950$

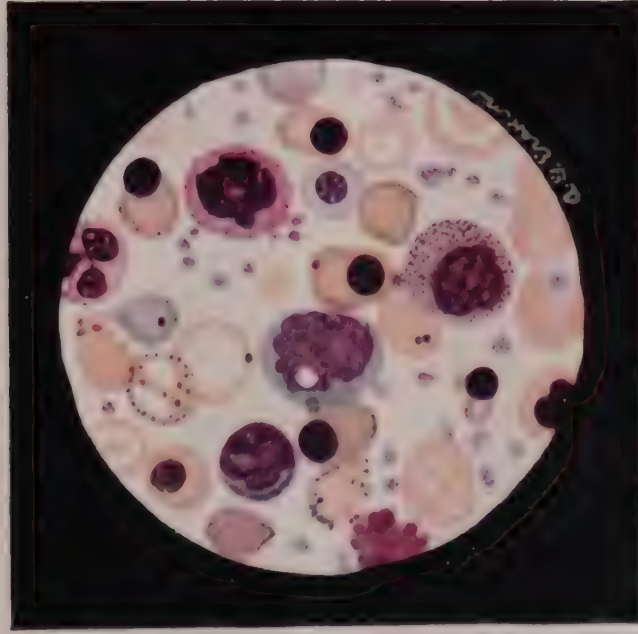
1. Marginal granule in reticulocyte
2. Reticulocyte
3. Normoblasts with stained reticulum
4. Reticulocyte with Howell-Jolly body
5. Normoblast
6. Howell-Jolly body
7. Fragmentary nuclear structure
8. Blood platelet
9. Poikilocyte
10. Target cells (1)
11. Hypochromic cells. Marked anisocytosis and hypochromia



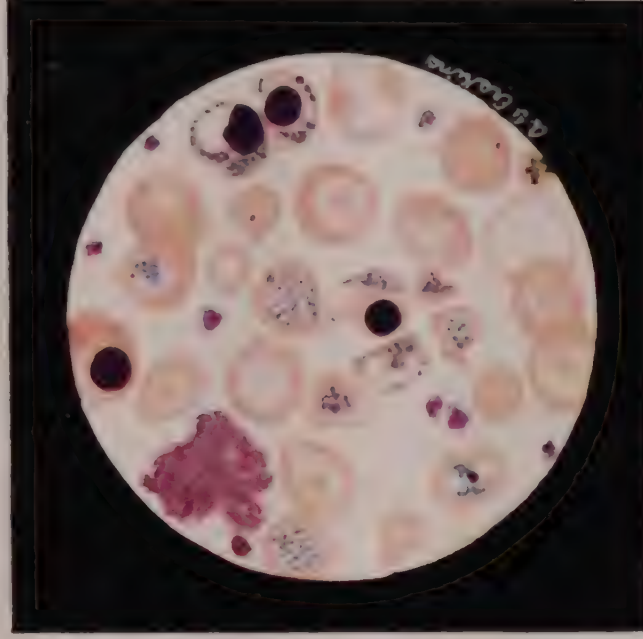
BONE MARROW
WRIGHT-GIEMSA STAIN $\times 950$

1. Erythrogonia
2. Normoblast
3. Normoblast in karyorrhexis
4. Howell-Jolly body
5. Polychromatic macroblast
6. Lymphocyte
7. Promyelocyte
8. Neutrophilic juvenile
9. Neutrophilic "stab"
10. Segmented neutrophile
11. Eosinophilic juvenile
12. Fragmentary nuclear structure
13. Polychromatic erythrocyte

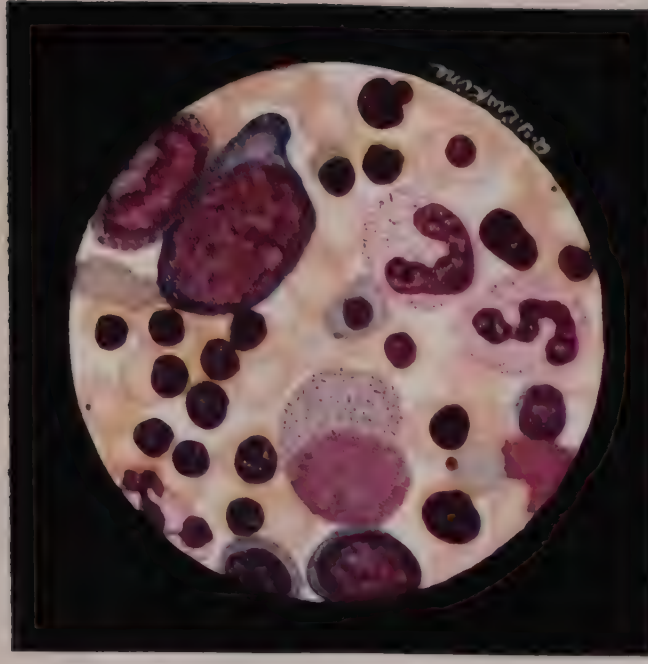
IDIOPATHIC HEMOLYTIC ANEMIA



BLOOD FILM
GIEMSA STAIN X950



BLOOD FILM-RETICULOCYTES
SUPRAVITAL STAIN X950



BONE MARROW
WRIGHT - GIEMSA STAIN X950

hemolytic transfusion reactions and hemolytic disease of the newborn. The type described by Lederer below is an acute type. The causes of the chronic hemolytic anemias are classified as sickle cell anemia (see pages 734 ff.), chronic or acquired hemolytic jaundice, Cooley's anemia, and cases found with other chronic diseases, as pernicious anemia, Hodgkin's disease, and carcinomatosis.

In general, free hemoglobin and other blood pigments are found in the blood plasma, and hemoglobinuria is seen. The chief pigment found is methemoglobin, described by Fairley.¹ Hemoglobinemia, methemalbuminemia, and bilirubinemia are detectable in the plasma in all hemoglobinurias, depending on the rapidity and severity of the blood destruction. The bone marrow in acute hemolytic anemias is hyperplastic. Anemia due to the plasmodium of malaria is the result of destruction of red cells. This is a normocytic anemia. In blackwater fever due to *Plasmodium falciparum*, methemalbumin is found in the plasma as well as bilirubin. The cause of blackwater fever, as seen in severe malarial infections, is not definitely known. Various theories have been offered to explain it. Some believe it is due to sensitization to protein antigens of the malarial parasite produced by earlier infections. In Bartonella infections there is acute blood destruction. The most serious type of Bartonella infections found in the tropics is called Oroya fever due to the *Bartonella bacilliformis*. The vector is the Phlebotomus. Verruga peruana is due to the same organism.

Acute Hemolytic Anemia.—This is a type of anemia which occurs in the first two decades of life. Lederer² described three cases characterized by sudden onset, elevation of temperature, leukocytosis, and severe anemia, resembling pernicious anemia, all of which disappeared following a single blood transfusion. He maintained that these cases, of a type hitherto undescribed in literature, were due to an infection with selective action concentrated on the reticuloendothelial system. These cases showed a remarkable acuteness of the development of symptoms, evidences of very rapid erythrocytic destruction, extreme regenerative action of the bone marrow, rapid recovery after therapy, and the absence of sequelae. Clinically, cases began with headaches, weakness, yellowness of the skin, rapid pulse, and elevated temperature. One of Lederer's cases showed superficial retinal hemorrhages, localized mainly about the discs. All the evidences of a profound anemia with every manifestation of hyperfunction of the bone marrow were seen. A very close resemblance of the blood in pernicious anemia during a crisis was noted. Red cells with mitotic, pyknotic, fragmented, degenerated, and freshly extruded nuclei were found. Megaloblasts were easily demonstrated. A high degree of anisocytosis and poikilocytosis, numerous polychromatic cells, and cells with basophilic granulation dominated the picture. A pronounced macrocytosis and microcytosis were noted, and most of the cells appeared hyperchromic. Lymphocytes were but slightly increased in proportion to the myeloid cells. The latter showed in small quantities every type of cell associated with myelocytic leukemia, some undifferentiated, and others in various stages of dif-

¹Fairley, N. H.: Quart. J. Med. 10: 95, 1941.

²Lederer, Max: Am. J. M. Sc. 170: 500, 1925.

ferentiation. All these findings point to the stimulating action of some irritant, resulting in hyperfunction of hemopoiesis affecting the leukopoietic and erythropoietic elements equally.

Moschcowitz² noted a case which he called "an acute febrile pleiochromic anemia with hyaline thrombosis of the terminal arterioles and capillaries." He called it an undescribed disease although its essential characteristics definitely suggest its similarity to the case of acute hemolytic anemia described by Lederer. In his patient, a girl of 16, the onset was sudden with a rise in temperature, a few petechiae on the arm, and a red blood count of 1,330,000, hemoglobin of 40 per cent, leukocytes 12,600, of which 65 per cent were polymorphonuclears. A fragility test showed hemolysis complete at 0.19. Urine showed albumin and hyaline and granular casts. Patient showed partial paralysis of the left arm and leg and a slight facial paralysis. Patient died fourteen days after the onset of the disease. A partial necropsy was done. The heart muscle showed thrombi in the terminal arterioles or capillaries. Vessels were plugged with hyaline masses. These thrombi were first described by Klebs,³ who noted them in extensive burns. Schmorl⁴ found them in the stomach and liver after abrine poisoning. Flexner⁵ showed that they arose from agglutinated red cells, proving this by injecting the highly agglutinative substance, ricin, into rabbits.

Chronic Hemolytic Jaundice.—This is a pure type with an increased degree of susceptibility to hemolysis by hypotonic salt solution compared to the normal erythrocytes. Some authors consider the acquired type of hemolytic jaundice a manifestation of a latent familial case. The acquired types develop after attacks of malaria, syphilis, and other infections, but also occur without evident cause. In the familial type, the predisposition is transmitted as a Mendelian dominant by either parent. In some cases there are transient mild attacks with very little anemia; in other cases, there are persistent jaundice and severe anemia of a fatal type. As Castle and Minot⁶ state, "In the acquired type the patient commonly is 'more sick than jaundiced' and in the congenital often 'more jaundiced than sick.' "

There are also seen other atypical hemolytic anemias, sometimes described as atypical instances of hemolytic jaundice and at other times as an idiopathic hemolytic anemia. Refer to the section on Immunohematology for further discussion.

The blood picture in these cases showed reticulocytes, leukocytes and blood platelets increased when the patients are definitely jaundiced. The red cell count is low but may become normal in remissions. The reticulocytes are above 10 per cent. Baty⁷ described a case with 92 per cent reticulocytes. Red cells are small. There is increased susceptibility of the cells to hemolysis by hypotonic salt solution. Plasma pigments are increased and the indirect van den Bergh reaction is positive.

²Moschcowitz, Eli: *Arch. Int. Med.* 30: 89, 1925.

³Klebs: *Handb. d. path. Anat.* Berlin 2: 114, 1868-1880.

⁴Schmorl: *Jahresb. d. Gesselsch. f. Nat.-u. Heilk. in Dresd.*, 1899-1900.

⁵Flexner: *J. M. Res.* 8: 316, 1902.

⁶Castle, Wm. B., and Minot, Geo. R.: *Pathological Physiology and Clinical Description of the Anemias*, Oxford University Press, 1936, page 35.

⁷Baty, J. M.: *Am. J. M. Sc.* 179: 546, 1930.

A case of idiopathic hemolytic anemia under observation in our service at the Christian Hospital, St. Louis, Mo., follows.⁸

The patient is a school girl, 9 years of age (1942). She had had a hemolytic anemia, unrelieved by splenectomy in 1934. Past diagnosis (before entrance into the Christian Hospital) was of Banti's syndrome.

Previous History.—The patient was admitted because of a severe rash, found to be scabies. Striking features of the case were the pasty, slightly cyanotic, and icteric color of the face. Enormous liver, extending 4½ inches below the costal margin in the nipple line; usual contour of the liver is preserved; there is no tenderness. Entire abdomen much distended, with flatness in both flanks. Kidneys are neither palpable nor elicited by percussion. Tonsils are in a poor condition. There are enlarged lymph glands in the neck region. Heart is enlarged to the left, the rate rapid and regular, with no murmurs.

Laboratory findings on admission were:

Fragility test: only very slight hemolysis beginning at 0.32%. Control hemolysis began at 0.40% and was complete at 0.34%.

Blood sedimentation: 6 mm. in 1 hour.

Stool: no blood, no leukocytes, no eggs or parasites.

Icterus index: 16.7.

van den Bergh: greatly delayed direct reaction, over 3 minutes; 1.4 units, indirect reaction.

Thrombocytes: absolute, 330,000 per cu. mm.; relative 106 per 1,000 erythrocytes.

Serology: negative.

X-ray: x-rays of the skull, ribs, spine, forearm, and both thighs reveal a marked increase in the total width of all bones. This is due to an increase in the medullary structures, with decrease in the size of the bone cortex. It is partially noted in the flat bones of the skull and pelvis. The spicule formation is typical of long-standing anemia, in which the bone marrow has assumed the production of excessive erythrocytes. *Conclusion:* Marked bone changes indicating erythroblastic anemia.

The blood picture and bone marrow counts on admission were as follows:

Blood:*

DATE	LEUKO-CYTES	ERYTHRO-CYTES	HGB. SAHLI	C.I.	B.	E.	M.	J.	ST.	SEG.	L.	MONO.	COAG. TIME	BLEED. TIME
4/17/40	27,150	3,280,000	40%	0.62	—	14	—	—	7	71	6	2	3'30"	2'45"
4/22/40	—	3,470,000	42%	0.61	—	3½	1	2½	9	41	38	4½	½ plasma cell (patient had been treated for scabies)	

For every 100 leukocytes, there were:

490 normoblasts

3 macroblasts

1 microblast

Many cells showed coarse basophilic punctation. There were large numbers of Howell-Jolly bodies, marked polychromasia, marked anisocytosis, with many hypochromic macrocytes, even hypochromic megalocytes, karyogenic metachromasia, karyorrhexis, marked hypochromia, many marginal granules, many poikilocytes. *Normocytic hypochromic erythrocytes with macrocytes.*

*See Plate XVI.

⁸Courtesy Dr. Robert Warner, St. Louis, Mo.

Bone Marrow:

Myeloblasts	3	For every 100 leukocytes, there were
Promyelocytes	10	2 erythrogenia
Eosinophilic myelocytes	1/2	15 p. megaloblasts
Eosinophilic "stabs"	1/2	23 p. macroblasts
Segmented eosinophiles	2 1/2	437 normoblasts
Basophiles	1/2	1 megaloblast with double nuclei, without signs of mitosis
Neutrophilic myelocytes	12 1/2	Coarse basophilic punctation, many Howell-Jolly bodies, marginal granules.
Neutrophilic juveniles	17	3 cells in mitosis
Neutrophilic "stabs"	19	
Segmented neutrophiles	25 1/2	
Lymphocytes	8 1/2	
Monocytes	1/2	
Plasma cells present		

A diagnosis of idiopathic hemolytic anemia was made. Patient now returns to the hospital every few months, remains about a week, and receives blood transfusions. Changes in the erythrocyte counts and hemoglobin percentages following the transfusions were:

Date	Erythrocytes	Hemoglobin, Sahli	Number of transfusions
7/29/40	1,950,000	28%	5—250 c.c. blood
8/ 3/40	3,000,000	52%	
10/14/40	2,480,000	38%	2—300 c.c. blood
11/17/40	3,150,000	51%	Leukocyte count: 21,400

The complete blood count, made before a series of transfusions, follows:

DATE	LEUKO- CYTES	ERYTHRO- CYTES	HGB.	C.I.	R.	E.	M.	J.	ST.	SEG.	L.	MONO.
7/13/41	18,750	3,040,000	48%	0.8	—	—	—	—	6	65	21	7

For every 100 leukocytes, there were:

- 1 polychromatic megaloblast
- 2 orthochromatic megaloblasts
- 1 polychromatic macroblast
- 203 polychromatic normoblasts
- 384 orthochromatic normoblasts, 63 with basophilic punctation, 1 with karyorrhexis
- 20 orthochromatic microblasts.

The total number of nucleated red cells was 611 per 100 leukocytes. Blood showed very marked anisocytosis and poikilocytosis; very marked hypochromia; marked basophilic punctation, both medium and coarse; slight polychromasia; many macrocytes; occasional orthochromatic and polychromatic megalocytes, all hypochromic; very many Howell-Jolly bodies; slight karyogenic metachromasia.

Blood count made in May, 1942:

LEUKO- CYTES	ERYTHRO- CYTES	HGB.	C.I.	R.	E.	M.	J.	ST.	SEG.	L.	MONO.
28,500	2,230,000	44% (S)	1.0	—	3	2	10	11	43	23	8

For every 100 leukocytes, there were:

- 91 polychromatic normoblasts
- 11 orthochromatic normoblasts
- 2 orthochromatic normoblasts in karyorrhexis
- 7 polychromatic microblasts
- 28 polychromatic macroblasts
- 18 polychromatic megaloblasts
- 147 nucleated reds, total

There was moderate anisocytosis and poikilocytosis, slight polychromasia, slight basophilic punctation (fine), many fragmentary nuclear structures, Cabot ring bodies, and Howell-Jolly bodies.

Thalassemia

Thalassemia is a hemolytic anemia associated with an inherited intracorpuseular defect of erythrocytes. First differentiated by Cooley and Lee,¹ it has often been referred to as "Cooley's anemia." This syndrome has also been designated "Mediterranean anemia" or "Mediterranean disease." The latter designation has been applied chiefly because the syndrome has occurred most frequently in people of Mediterranean stock, with Italians predominating. Although scattered cases had been described in non-Mediterranean stock, it was not until Minnich and her co-workers² reported 32 cases in Thailand that any considerable number had been collected outside of this group. In subsequent studies, most of these cases were found to be a combination of Mediterranean-E hemoglobin disease rather than pure thalassemia.³ It is quite generally agreed today that the syndrome is due to an inherited characteristic of the erythrocyte⁴ as a recessive gene. Two major subdivisions are recognized: (a) the symptomatic disease form, thalassemia major, and (b) an asymptomatic form, thalassemia minor. Thalassemia major is the homozygous disease form with a double dose of the gene for the syndrome, whereas thalassemia minor is the heterozygous trait form, having only one of the genes for Mediterranean disease. In addition to these major divisions, studies of electrophoresis and alkali denaturation have revealed combinations of thalassemia and molecular disease of hemoglobin. Examples of such combinations are sickle-thalassemia disease, thalassemia-C disease, and thalassemia-E disease (see section on molecular disease of hemoglobin, pages 734 ff.). The combination of sickle-thalassemia is sometimes referred to as "microdepranocytosis."

In the disease form, anemia is a striking feature, varying in intensity from case to case, but often of sufficient severity to cause symptoms. An outstanding feature of the disease is the x-ray appearance of the skull which shows perpendicular striations suggesting hair standing on end. Involvement of other bony structures may be present, and in the hands it takes on a mosaic pattern. Splenomegaly may occur and at times reaches fairly high proportions. The hemolytic crises are often attended by febrile periods but jaundice is infrequent. As a result of the anemia, debility, and perhaps a lessened resistance, intercurrent infections are fairly frequent and life expectancy is considerably reduced, so that many of these patients do not live beyond childhood.

The blood picture in the disease form shows an anemia of variable degree, ranging from 1,000,000 up to 3,500,000 red cells per cu. mm. There is a pronounced lowering of the hemoglobin and hematocrit values, with a resultant hypochromic microcytic anemia. The blood spread not only reflects this hypochromasia and microcytosis but also shows marked poikilocytosis and anisocytosis. During and shortly following a hemolytic crisis, nucleated erythrocytes

¹Cooley, T. B., and Lee, P.: *Tr. Am. Pediat. Soc.* 37: 23, 1925.

²Minnich, V., NaNakorn, S., Chongchareonsook, S., and Kochasani, S.: *Blood* 9: 1, 1954.

³Personal communication of Dr. Amos I. Chernoff.

⁴Valentine, W. N., and Neel, J. V.: *Am. J. M. Sc.* 215: 456, 1948.

are commonly seen in the blood spreads and Howell-Jolly bodies may be present. Target cells in varying numbers are so regularly encountered that their presence in a blood spread should always suggest thalassemia as a possible diagnosis. Reticulocytes are often increased. There are no significant changes in the platelets. The leukocyte count may be considerably elevated during a period of hemolytic crisis. The bone marrow is usually hyperplastic, displaying a normoblastic hyperplasia, which is not a specific characteristic enabling a diagnosis of the disease.

Fragility studies of the erythrocytes show either a normal osmotic fragility or may show an increased resistance in hypotonic saline. Red cell survival studies reveal a diminished survival time in some cases and a normal survival in others.⁵ The observations of Singer and associates⁶ that thalassemia major displays a considerable increase in hemoglobin F have been verified by many observers.

In thalassemia minor, there are no symptoms referable to the disorder. Although anemia is not present, target cells may be found in the blood spreads in lesser numbers than in the disease form. The same is true of the amount of fetal (F) hemoglobin.

In the combinations of thalassemia and sickle cell hemoglobin C or hemoglobin E, the clinical and blood pictures appear to have a position intermediate between thalassemia disease and thalassemia trait. Although sickling is not present in thalassemia alone, when it combines with the sickle form, sickling of the red cells is demonstrable. Electrophoretic studies in these combinations demonstrate the abnormal hemoglobin S, C, or E, as well as F. However, attention is again called to the fact that hemoglobin F has a mobility approximating that of A and cannot be readily demonstrated by paper electrophoresis.

Paroxysmal Nocturnal Hemoglobinuria

Paroxysmal nocturnal hemoglobinuria, often referred to as the Marchiafava-Micheli syndrome, is associated with a chronic hemolytic anemia with acute paroxysms of hemolytic crises occurring at night. These crises are of sufficient degree to result in hemoglobinuria. Although the red cell is the predominant hemopoietic element involved, the white cells and platelets may also be affected. Crosby⁷ has presented a magnificent review of this syndrome, together with many of his original observations. He concludes that paroxysmal nocturnal hemoglobinuria is an acquired disease of the hemopoietic system in which abnormal red cells, white cells, and platelets are then susceptible to some normal plasma factor, which is believed to be properdin.⁸ This serum euglobulin is distinct from complement and the components of the clotting system but requires complement and magnesium ion for its activity. Crosby calls attention to the fact that hemolytic crises occur when the patient is asleep (whether at night or day), and that during this period changes in the balance of an inhibitor-hemolysin system may account for the crises. Others

⁵Kaplan, E., and Zuelzer, E. W.: *J. Lab. & Clin. Med.* 36: 519, 1950.

⁶Singer, E., Chernoff, A. I., and Singer, L.: *Blood* 6: 413, 1951.

⁷Crosby, W. H.: *Blood* 8: 769, 1953.

⁸Hinz, C. F., Jordan, W. S., and Pillemer, L.: *Proc. Central Soc. Clin. Res.* 27: 57, 1954.

have felt that changes of pH are produced by retention of CO₂ during sleep; this observation forms the basis of the Ham¹ acidification test and its modifications.

In addition to the anemia, these patients develop leukopenia, which may become chronic, and perhaps contributes to the increased susceptibility to infection that these patients show.

Thrombocytopenia is generally not marked and purpura is uncommon. However, agglutination of platelets in this disease accounts for the vascular thromboses seen in paroxysmal nocturnal hemoglobinuria. There appears to be some evidence of a vicious cycle of competition between members of the coagulation mechanism (especially thrombin) and the factors involved in paroxysmal nocturnal hemoglobinuria. Dicumarol has had some beneficial effect in protecting these patients against thrombotic episodes and perhaps the development of anemia. Heparin, on the other hand, causes increased hemolysis.

Paroxysmal Cold Hemoglobinuria

This condition is found in syphilis and is often acutely brought on by sudden chilling of the body surface. The urine is diagnostic; if taken directly after or during the attack, color varies from brown red to black; acid reaction; specific gravity very low; on boiling, a brown coagulum of albumin develops; hyaline casts are present; spectroscopically, bands of hemoglobin and methemoglobin; there are no red cells in the urine; urine contains urobilin.

During the attack we may find the following *Donath-Landsteiner auto-hemolysis phenomenon*: Place some blood in a test tube. Chill to 5° C. for ten minutes. Warm to 37° C. Hemolysis will occur. Normal blood will show no such hemolysis. This test is based upon the fact that in this disease a special hemolytic substance has been liberated into the blood stream. We note, too, in the blood picture, leukopenia, hypochromia, and poikilocytosis during the attack.

Blood Picture in Bartonellosis

The blood picture in bartonellosis, according to Groot,² shows a rapid, progressive anemia. Erythrocyte counts of 1,000,000 per cu. mm. are common. The rate of decrease is rapid, with as many as 300,000 red cells per cu. mm. lost in 24 hours.

The volume, diameter, and thickness of the erythrocytes is greater than normal. The mean hemoglobin concentration is usually low, but may be normal. Hyperbilirubinemia is often observed, but is not constant. The bone marrow is regenerative, with large numbers of reticulocytes and normoblasts in the peripheral blood, the number of such elements being proportional to the seriousness of the anemia. Megaloblasts are often found.

Bartonellae are found in the red blood cells, the number varying. At times, 99 per cent of the red blood cells are infected, and often it is possible to find 10 to 15 organisms in a single cell, although these are extreme cases. Basophilic erythrocytes and macrocytes are usually less infected. Reticulo-

¹Ham, T. H.: A Syllabus of Laboratory Examinations in Clinical Diagnosis, Cambridge, Mass., 1950, Harvard University Press, p. 167.

²Groot in Clinical Tropical Medicine, by Gradwohl, Benitez Soto, and Felsenfeld; St. Louis, 1948, The C. V. Mosby Co.

cytes seldom are infected unless the number of Bartonellae in the blood is very high. Normoblasts are seldom infected except in rare cases. Bartonellae are sometimes seen in the cytoplasm of monocytes.

At the beginning of the disease, there is leukopenia, although the number of white blood cells is normal in uncomplicated cases.

There is no typical differential count. At times either a very slight lymphocytosis is noted or, more commonly, polymorphonucleosis.

During the period of anemia, there is an increase in young polymorphonuclears, etc., in the myeloid series.

The sedimentation rate is greatly increased, 100 to 172 mm. in one hour being noted in the Westergren test.

Autoagglutination phenomena are rare.

According to Schilling, this is the only acute infection which causes a severe anemia of the megalocytic type.

Molecular Disease of Hemoglobin

Pauling's¹ discovery of the differences in electrophoretic mobility of normal and sickle cell hemoglobin has opened a rapidly growing field in the investigation of hemoglobin. The basic concept of this work is that hemoglobin exists in various molecular structures that differ from that of normal hemoglobin. These differences can be elucidated by electrophoresis, as well as by other methods such as gel formation,² tactoid formation in phase microscopy,^{2, 3, 7} and alkali denaturation.⁴ The technic of paper electrophoresis for studying proteins, including hemoglobin,^{5a, 5b} has supplemented the much more complicated and expensive Tiselius apparatus, and has opened the way to large-scale investigation of diseases associated with hemoglobin molecular disease.

The **nomenclature⁶ of the various types of hemoglobin** now described on the basis of electrophoretic patterns are as follows:

(a) Normal adult hemoglobin, or hemoglobin *A*; (b) normal fetal hemoglobin, or hemoglobin *F*⁴; (c) sickle cell hemoglobin, or hemoglobin *S*⁹; (d) hemoglobin *C*^{9, 10, 11}; (e) hemoglobin *D*^{8, 9}; (f) hemoglobin *E*¹² and an unnamed hemoglobin¹³ which appears to have a position intermediate between *F* and *S*.

These hemoglobin types are inherited. Neel¹⁴ has reported the inheritance pattern of the sickling phenomenon in 75 kindreds. He maintains that this phenomenon is "due to a gene which in single dose (heterozygous condition) produces only the sickle cell trait, and in double dose (homozygous condition)

¹Pauling, L., Itano, H. A., Singer, S. J., and Wells, I. C.: *Science* **110**: 543, 1949.

²Harris, J. W.: *Proc. Soc. Exper. Biol. & Med.* **75**: 197, 1950.

³Bull, H. B.: *Physical Biochemistry*, New York, 1951, Wiley and Sons, p. 246.

⁴Singer, K., Chernoff, A. I., and Singer, L.: *Blood* **6**: 413, 1951; *ibid.* **6**: 429, 1951.

⁵(a) Kunkel, H. G., and Tiselius, A.: *J. Gen. Physiol.* **35**: 89, 1952.

(b) Spaet, T. H.: *J. Lab. & Clin. Med.* **41**: 6, 1953.

⁶Statement concerning a system of nomenclature for the varieties of human hemoglobin. *Blood* **8**: 386, 1953.

⁷Roche, J., Derrien, Y., and Roques, M.: *Compt. rend. Soc. de Biol.* **146**: 680, 1952.

⁸Itano, H. A.: *Science* **117**: 89, 1953.

⁹Itano, H. A.: *Proc. Nat. Acad. Sc.* **37**: 775, 1951.

¹⁰Kaplan, E., Zuelzer, W. W., and Neel, J. V.: *Blood* **6**: 1240, 1951.

¹¹Gray, G. W.: *Scient. Am.* **185**: 56, 1951.

¹²Chernoff, A. I., Minnich, V., Chongcharoensook, S., NaNakorn, N., and Chernoff, R.: *Proc. Central Soc. Clin. Invest.* **27**: 26, 1954.

¹³Battle, J. D., Jr., and Lewis, L.: *Proc. Central Soc. Clin. Invest.* **27**: 11, 1954.

¹⁴Neel, J. V.: *Blood* **6**: 389, 1951; *ibid.* **7**: 467, 1952.

sickle cell disease." Translated into terms of abnormal hemoglobins, patients with sickle cell trait are represented by an S-A gene pattern, whereas those with sickle cell disease have an S-S pattern. The same principle applies to other combinations of abnormal hemoglobins. In the trait, quantitative

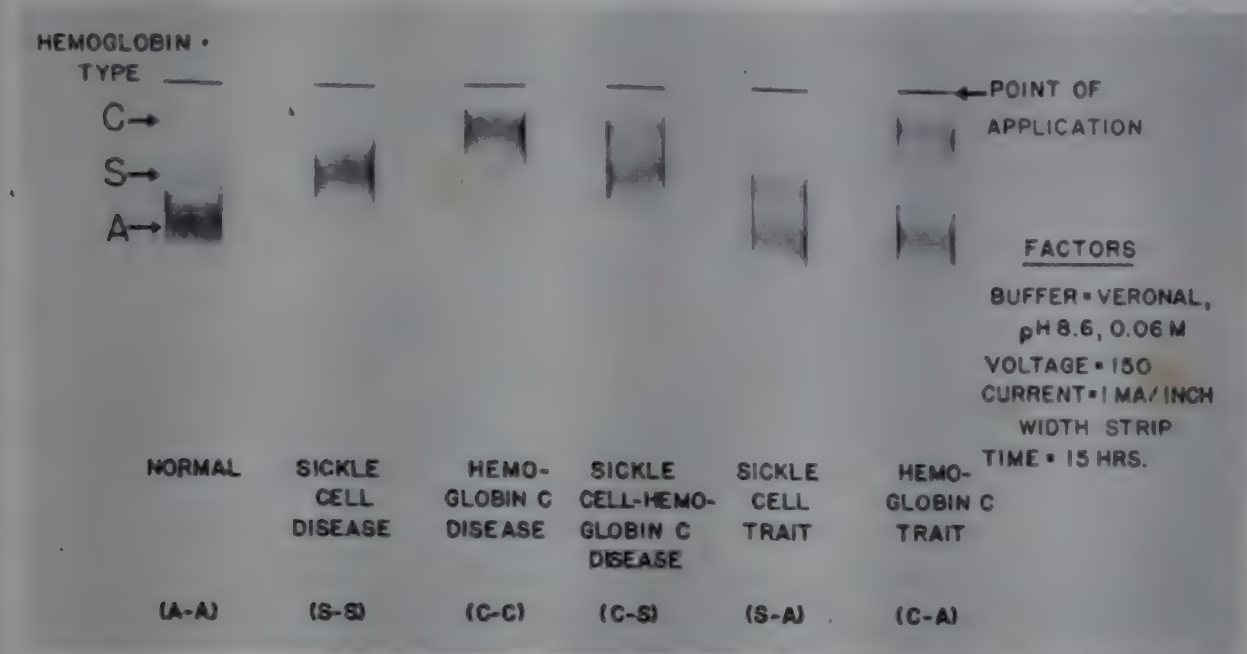


Fig. 194.—Electrophoretic determination of hemoglobin types. Separation of hemoglobins by filter paper electrophoresis. (Weinstein, I. M., Spurling, C. L., Klein, H., and Neeheles, T. F.: *Blood* 9:1157, 1954. By permission of the authors and publisher.)

electrophoretic studies show small amounts of abnormal hemoglobin in combination with relatively large amounts of normal hemoglobin. On the other hand, the disease syndrome reveals almost 100 per cent abnormal hemoglobin. Motulsky and his co-workers¹⁵ report the following typical distribution of

TABLE 53.—DISTRIBUTION OF VARIOUS TYPES OF HEMOGLOBIN

	HEMOGLOBIN TYPE					
	A	S	C	D	E	F
Normal newborn	15-50%	—	—	—	—	50-85%
Normal child up to age 2½ years	97-100%	—	—	—	—	Up to 3%
Normal adult	98.4-100%	—	—	—	—	Trace
Sickle cell trait	55-76%	24-45%	—	—	—	Trace
Sickle cell disease	—	75-100%	—	—	—	0-25%
Hemoglobin C disease	—	—	100%	—	—	Trace
Hemoglobin C trait	60-75%	—	25-40%	—	—	Trace
Sickle-C disease	—	33-50%	50-67%	—	—	Less than 2%
*Thalassemia-C disease	25%	—	75%	—	—	Trace
Hemoglobin D trait	51-65%	—	—	35-49%	—	—
Sickle-D disease	—	?	—	?	—	?
Thalassemia minor	90-100%	—	—	—	—	0-10%
Thalassemia major	0-60%	—	—	—	—	40-100%
Sickle-thalassemia disease	18-33%	67-82%	—	—	—	1-17%
†Hemoglobin E trait	++++	—	—	—	+	—
†Hemoglobin E disease	—	—	—	—	94-98%	2-6%
†Thalassemia E disease	—	—	—	—	60-80%	20-40%

*a. Singer, K., Kraus, A. P., Singer, L., Rubinstein, H. M., and Goldberg, S. R.: *Blood* 9: 1032, 1954.

b. Zuelzer, W. W., and Kaplan, E.: *Blood* 9: 1047, 1954.

†Chernoff and associates.¹²

¹⁵Motulsky, A. G., Paul, M. H., and Durrum, E. L.: *Blood* 9: 897, 1954.

hemoglobins in various syndromes observed by them or collected from the literature. Table 53 is a modification, based on observations made subsequent to their report.

The electrophoretic mobilities of the various hemoglobins follow the order of $A > F > E > S(D) > C$. The mobility of hemoglobin F so closely approaches the speed of hemoglobin A that these two components are generally inseparable in the paper electrophoresis technic, although Smith and Conley¹⁶ claim such separation is possible. In the Tiselius apparatus, the F component can be readily identified. In most clinical studies, the alkali denaturation technic⁴ has been the method of choice for demonstrating F-hemoglobin. D-hemoglobin has the same mobility as S-hemoglobin and cannot be separated by electrophoresis. Differences in solubility⁹ have been used as a means of distinguishing these two components.

The presence of these various abnormal hemoglobins may or may not result in symptoms. In general, patients with a homozygous pattern and high percentages of abnormal hemoglobin show marked symptoms. Hemolytic crises with resultant anemia are prominent features. Associated with these crises are severe abdominal pain, fever, headache, hematuria, pallor, tachycardia, and dyspnea. Abdominal pain may be so severe as to suggest a surgical condition of the abdomen, and patients in this category have been mistakenly operated upon as emergencies. Jaundice, as a result of excessive red blood cell destruction, is quite common, and hematuria is found occasionally. Vascular thromboses may result in a multiplicity of symptoms with the brain a fairly common site for such episodes. On physical examination, the degree of pallor is proportionate to the anemia. Jaundice may be present. Splenomegaly and sometimes hepatomegaly are quite common. In the heterozygous state, abnormal clinical findings are absent and the patient's history is essentially negative. In combined states, such as sickle-C hemoglobin disease, the clinical picture is generally intermediate in severity between the homozygous and heterozygous conditions. Although the picture is generally milder in such combinations, Smith and Conley¹⁶ call attention to two patients in whom hemolytic crises were sufficiently severe to produce jaundice and profound anemia.

Just as the clinical pattern seems to follow the types of hemoglobin present, there also appears to be some parallelism in the hematologic studies. In the *homozygous* states, generally, an anemia of varying degree occurs and may be quite profound. The stained blood spreads show numerous target cells, ranging from 60 to almost 100 per cent of the red blood cells. During crises, anisocytosis (chiefly microcytosis) and poikilocytosis are marked, and nucleated erythrocytes are common. Polychromasia is often seen. In those patients with sickle cell disease, sickled erythrocytes may rarely be seen on the blood spread. Osmotic fragility studies reveal an increased resistance. Mechanical fragility tends to be normal in an oxygen atmosphere but is increased in an atmosphere saturated with carbon dioxide. Reticulocytes are often increased, especially during or shortly after a hemolytic crisis. Siderocytes, which are iron-staining bodies within the erythrocytes, tend to be numerous.¹⁷ Sickling is dem-

¹⁶Smith, F. W., and Conley, C. L.: Bull. Johns Hopkins Hosp. 93: 94, 1953.

¹⁷Kaplan, E., Zuelzer, W. W., and Neel, J. V.: Blood 8: 735, 1953.

onstrated only in patients with hemoglobin S and may approximate 100 per cent in some instances. In this respect, it is important to point out that sickling of such proportion occurs only in the homozygous S state. Homozygous C and E and thalassemia are not accompanied by sickling phenomenon. When these are combined with sickling such as sickle-C hemoglobin disease, sickling will occur but to a lesser degree than in homozygous S.

Combinations of sickle cell and other abnormal hemoglobins, such as sickle-C hemoglobin disease, show intermediate hematologic values. Kaplan, Zuelzer, and Neel¹⁷ were particularly impressed with the markedly high target cell incidence in sickle-C hemoglobin disease. Apparently variations in hematologic abnormalities may show the same wide variations that the clinical pictures display, ranging from relatively mild to very severe.

In the *heterozygous* or *trait* states, hematologic studies reveal minimal deviations from normal. Anemia is not present. Target cells occur much more frequently than in normal people but the percentage of such cells rarely exceeds 20 per cent. Osmotic fragility tends to be normal or occasionally decreased, and mechanical fragility is generally normal or only slightly affected in CO₂. Siderocytes are absent or rare. Sickling in wet preparations is minimal, rarely exceeding 20 per cent in subjects with S-hemoglobin.

From a clinical standpoint, the relationship of F-hemoglobin concentration to disease is not quite as clear-cut as with the other abnormal hemoglobins. Its high concentration in newborn infants (almost 100 per cent) is not implicated in any disease process. Singer and his co-workers⁴ originally demonstrated its association with sickle cell disease where values ranging from 2.0 to 23.9 per cent were found. Since this original publication, denaturation studies have taken on a pattern. Sickle-C disease and sickle-thalassemia disease are both associated with high values for fetal hemoglobin. Thalassemia major is also associated with increased F-hemoglobin values. Mild elevation of F-hemoglobin has been noted in E disease. When thalassemia is combined with other abnormal hemoglobins (e.g., thalassemia-E disease), fetal hemoglobin values are elevated. In the trait states of S-hemoglobin or thalassemia minor, the values are normal. Increased amounts of F-hemoglobin have been noted in some miscellaneous conditions, such as hereditary spherocytosis, chronic aregenerative anemia, untreated pernicious anemia, myelophthisic anemia, and some leukemias.

Sickle Cell Anemia

This form of anemia was first seen by Herrick¹ who observed peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia. He did not recognize this as a distinct clinical entity, but it is now recognized as such. The disease is a familial one occurring mainly in Negroes or mulattoes. It was generally believed that it was an intrinsic characteristic of the Negro, until Cooley and Lee² reported a case in a four-year-old American-born Greek boy. Since then, other cases have been reported in Italians, Sicilians, and even in a boy of Scottish-Irish parentage. It has been found as a latent

¹Herrick, J. B.: Arch. Int. Med. 6: 517, 1910.

²Cooley, T. B., and Lee, P.: Am. J. Dis. Child. 32: 334, 1926.

phenomenon in 6 or 7 per cent of several series of supposedly normal Negroes. These cases show clinically a greenish yellow scleral discoloration. L. W. Diggs, of the University of Tennessee, has made a special study of this type of anemia. He states that the average age of death of these patients is eleven years, showing its high incidence in the young.

In West Africa, the ancestral home of most Negroes, sickle cell anemia received attention of Russell and Taylor in 1932, Smith in 1934, Reid in 1936, and Evans in 1944 and 1945. Findlay, Robertson, and Zacharias¹ reported on the incidence of sickle cell anemia in the British West African colonies. The technic was simple owing to field conditions. After cleaning the skin, a drop of blood was placed on a glass slide, covered with a clean cover glass, and ringed with petrolatum. Ringed preparations, protected from ants, were kept at room temperature and examined after 24 hours. In all, 5,500 Africans were examined, of whom 12.4 per cent showed the sickling trait. One-half of the number were natives of the Gold Coast and French Togoland, the remainder came from Gambia, Sierra Leone, and Nigeria. Sickling was found in all tribes and no significant specific tribal differences were noted; 11.2 per cent were males and 12.6 were females. They suggested that the expectation of life in those with sickling of the cells may be less than in normal persons. They investigated the incidence of sickle cell anemia in relation to certain physiologic and pathologic conditions, for instance, in pregnancy. Of 455 pregnant women examined, 77, or 16.9 per cent, showed sickling.

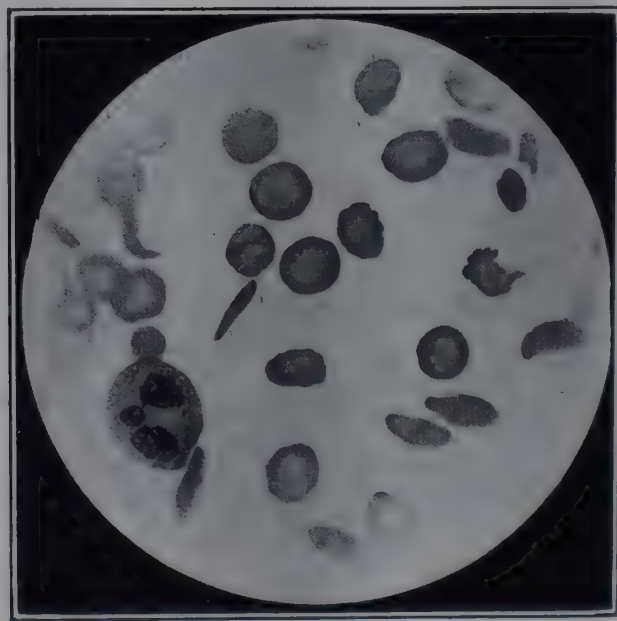


Fig. 195.—Sickle cell anemia, stained film. ($\times 950$.)

Sterility.—Of 61 women who either had never conceived or had failed to produce a live fetus, thirteen, or 21.3 per cent, showed the sickling trait.

Lunacy.—The rate of 6.5 per cent is comparable to that in old people without mental disease.

General Morbidity.—Fifteen and eight-tenths per cent (15.8) showed sickling.

Pneumonia.—It was attempted to determine whether or not jaundice seen in Negroes with pneumonia can be correlated with sickle cell anemia. Five and two-

¹Findlay, G. M., Robertson, W. M., and Zacharias, F. J.: *Tr. Roy. Soc. Trop. Med. & Hyg.* 40: 83-86, Aug., 1946.

tenths per cent (5.2) of West African soldiers who had pneumonia had jaundice. Of these pneumonia patients, 1,240 in all, 125 had sickle-shaped cells, and of the 125 with sickling, twelve, or 9.4 per cent, had jaundice. Difference in the incidence of jaundice in those with sickling and those without sickling is not particularly significant. In this communication, sickling in races other than Negroes was looked for with the following result—568 British soldiers were all negative and 188 Syrians were also all negative.

Blood Picture.—

The blood picture in sickle cell anemia shows sickle-shaped erythrocytes, often not typical in stained spreads, but 75 to 100 per cent are distorted in moist preparations. This sickling has often been observed in isotonic salt solution. Sydenstricker* concluded that the presence of serum is essential for this change in the cells. There is present a normocytic normochromic anemia. Signs of regeneration in the blood are seen in an increase in reticulocytes, diffuse basophilia, the presence of nuclear particles, nucleated red cells, and megalo-blasts. We have here anisocytosis, anisochromia, and poikilocytosis.

The differential blood count is variable, usually with a left shift of the leukocytes. There is also an increase in blood platelets. Bleeding and coagulation times are normal. There is a decrease in fragility, but normal sedimentation.

Demonstration of Sickling of Erythrocytes

Method I, In Vivo Anoxia, Scriver and Waugh¹

Place a rubber band around the finger for 5 minutes.

Obtain a drop of blood from the finger and quickly place it under a coverslip.

Seal the edges of the cover slip with petrolatum.

Observe for sickling for several hours.

Method II, Metabisulphite Method of Daland and Castle²

Add 1 drop of blood

to 2 drops of a freshly prepared 2% aqueous solution of sodium metabisulphite on a glass slide.

Cover with a cover slip and observe in 15 to 30 minutes.

With this method, sickling occurs promptly. In sickle cell *disease*, 60 to 80% of the red cells may show sickling, whereas in sickle cell *trait* only a small percentage (5%) may show sickling.

Bone Marrow Findings.—

Bone marrow studies in sickle cell anemia have not been touched upon with much frequency. Sydenstricker³ found the bone marrow to be abundant, bright red in color, and thin in consistency. Huck⁴ and Sharp and Schleicher⁵ confirmed these observations. In addition, the latter compared various quali-

*Sydenstricker, V. P., Mulherin, W. A., and Houseal, R. W.: *Am. J. Dis. Child.* 26: 132, 1923.

¹Scriver, J. B., and Waugh, T. R.: *Canad. M. A. J.* 23: 375, 1930.

²Daland, G. A., and Castle, W. B.: *J. Lab. & Clin. Med.* 33: 1082, 1948.

³Sydenstricker, V. P.: *J. A. M. A.* 83: 12, 1924.

⁴Huck, J. G.: *Bull. Johns Hopkins Hosp.* 34: 335, 1923.

⁵Sharp, E. A., and Schleicher, E. M.: *Am. J. Clin. Path.* 6: 580, 1936.

tative and quantitative studies on bone marrow and peripheral blood of an adult Negro with similar observations made on an adult Negress. Their differential counts are shown in Table 54.

TABLE 54.—DIFFERENTIAL CELL COUNTS ON BONE MARROW OF TWO CASES OF SICKLE CELL ANEMIA

CELLULAR ELEMENTS	PATIENT 2	PATIENT 3
	♀	♂
<i>Leukocytes</i>	<i>per cent</i>	<i>per cent</i>
Leukoblasts	3	3
Premyelocytes	7	10
Myelocytes, eosinophilic	3	4
Myelocytes, basophilic	1	1
Myelocytes, neutrophilic	13	18
Metamyelocytes, neutrophilic	17	23
Neutrophile, stab	20	18
Neutrophile, segmented	25	15
Eosinophiles	5	2
Basophiles	1	1
Lymphocytes	3	4
Monocytes	2	1
<i>Erythrocytes</i>		
Reticulocytes	15.8*	21.2*
Megalóblasts	12.0	3.2
Normoblasts	40.0	12.8
Mean diameter	7.8 μ	7.8 μ

*Reticulocytes given in per cent of erythrocytes and were counted separately after supravital staining.

The peripheral blood patterns of these two patients during their anemia phase were also compared with that of an adolescent Negress with sickle cell anemia. Their hematologic observations on their second patient are found in Table 55.

TABLE 55.—PATIENT 2. PERIPHERAL BLOOD
(Negress, 26 years of age, third trimester of pregnancy)

Erythrocytes	940,000 per cu. mm.
Mean diameter	8.1 microns
Thrombocytes	110,000 per cu. mm.
Reticulocytes	20.2%
Hemoglobin	25.0% (Newcomer)
Bleeding time	4.5 minutes
Coagulation time	3.0 minutes
Fragility	0.38 to 0.28% sodium chloride
Leukocytes	11,460 per cu. mm.
Myelocytes	1%
Metamyelocytes, neutrophilic	2%
Neutrophiles, stab	26%
Neutrophiles, segmented	49%
Eosinophiles	1%
Basophiles	1%
Lymphocytes	18%
Monocytes	2%

Among 100 leukocytes were found 28 per cent megaloblasts, 44 per cent normoblasts, and 12 per cent microblasts. The reticulocytes were predominantly young types showing heavy wreath forms of reticulation. There were marked anisocytosis, poikilocytosis, and polychromasia. Many stippled erythrocytes were found along with a small number of ovalocytes. The

thrombocytes varied in size and appeared toxic. The neutrophilic series showed a severe left shift of a degenerative type. There were many toxic granules.

In experimental work they showed that the sickling trait of the erythrocytes can be overcome by washing in saline and reinduced by addition of the patient's blood serum.

Sickling occurs in bone marrow cells of sickle cell anemia when sealed in normal serum from an individual of the same blood type. Blood cells from a normal individual of the same blood type retain their normal contour when in contact with serum from a case of a sickle cell anemia for a period of six days. The offspring of a patient showing severe sickle cell anemia did not manifest the sickling trait immediately after birth.

Pathology.—Sickled red blood cells; congested capillaries. There are a thrombocytic tendency, infarcts, fibrosis, anemia; and degenerative visceral changes are noted in the organs. There is hemolytic jaundice with pigmentation. The marrow is hyperplastic with evidence of accelerated hematopoiesis of all elements within the bone marrow. Unusual forms of red cells are found in specimens of bone marrow which have been prepared in Formalin. The lymph glands show marked hyperplasia. A number of observers have noted phagocytosis of red cells by reticuloendothelial cells in liver, spleen, and lymph glands. There are congestive splenic enlargement and early hemorrhage. There are siderotic nodules in the spleen. Fibrotic atrophy occurs late, as has been described by Diggs. There are hepatomegaly and some evidences of kidney degeneration. Leg ulcers are commonly seen.

Among other *laboratory findings*, there are the following: Hyperbilirubinemia; negative direct van den Bergh test; urobilinuria; albuminuria and casts; gastric hyperacidity; dark stools; roentgen ray changes in bones.

According to Diggs, the sickle cell trait is noted as follows: Best demonstrated by moist preparations; dominant Mendelian characteristic: 8 per cent incidence in Negroes; incidence in whites—rare authentic cases confined to the Mediterranean area.

The pathologic physiology of sickling was undetermined until Pauling demonstrated that the hemoglobin of sickle cell anemia (S-hemoglobin) had an electrophoretic mobility different than that of normal hemoglobin. He postulated this condition as a "molecular disease of hemoglobin." Many studies have verified and amplified these observations and they are covered in this edition under the section, "Molecular Disease of Hemoglobin," pages 734 ff.

Eadie and Brown¹ have critically reviewed the literature on red cell survival studies. In sickle cell anemia, it is known that when blood from a patient with the disease is transfused into a normal recipient, cells disappear rapidly at first and then more slowly with some cells living out a normal life span. The shortened survival time has been generally considered a function of the amount of abnormal hemoglobin in individual cells, those having the highest percentage of hemoglobin S having the shortest survival time. These

¹Eadie, G. S., and Brown, I. W., Jr.: *Blood* 8: 1110, 1953.

observers take exception to this view and suggest that random mechanical destruction plus normal senescence account for the disappearance rate in sickle cell disease.

Neel² has frequently been associated with discussions relating to the inheritance of sickle cell disease as a Mendelian dominant. He postulates a homozygous combination of genes as responsible for sickle cell anemia and a heterozygous combination as the picture in trait. In families in which sickle cell disease is found, both parents have the trait and 1 out of 4 children on the average inherits the pathologic gene from both parents to develop the disease. To date this is the group most frequently studied. As the new forms of molecular diseases of hemoglobin are uncovered, the problem becomes more complex. Combinations of sickle cell with hemoglobin C have been described and other combinations will undoubtedly be forthcoming. This second type has a lower frequency than sickle cell disease, and Neel indicates that in 6 per cent of Negro families in which sickle cell disease was present, the disease was due to a combination of sickle cell and hemoglobin C. The third type is a combination of sickle cell with thalassemia, which accounts for many of the cases of sickle cell disease in Caucasians. In these combinations, a gene for sickling is derived from one parent and the gene for hemoglobin C or thalassemia comes from the other parent. Silvestroni and Bianco³ call attention to the combination in which one parent has sickle trait and the other thalassemia trait; the offspring may have a hemolytic disease which they term "microdepranocytosis." On the basis of genetic studies in 11 families, these authors are of the opinion that the genes for sicklemia and microcythemia (thalassemia) are inherited independently of one another and are located on different chromosomes.

Differential Diagnosis.—One must differentiate this disease from acute arthritis, osteomyelitis, acute abdominal conditions, organic heart disease, catarrhal jaundice, septic fever, syphilitic or varicose leg ulcers, and hemolytic anemias. The blood picture will usually put one on the right track for a correct diagnosis. In these cases the anemia is a hemolytic one. The cells do not show more fragility than is seen in normal cells.

The average age at death, according to Diggs, is eleven years. Life expectancy is less than thirty years. Death is usually due to a complication, such as tuberculosis, pneumonia, or sepsis.

Ovalocytosis

Ovalocytosis is a familial phenomenon characterized by large numbers of oval or elliptical red cells, which appears as a non-sex-linked Mendelian characteristic in certain individuals. The etiology is unknown. To date seventy-five authentic cases and twenty-five questionable cases have appeared in the literature. Dresbach⁴ described a patient in whom every red cell was of oval shape; the blood was otherwise normal. Bishop⁵ described a case in whom 75 per cent of the erythrocytes were oval, with the blood otherwise normal.

²Neel, J. V., Itano, H. A., and Lawrence, J. S.: *Blood* 8: 434, 1953.

³Silvestroni, E., and Bianco, I.: *Blood* 7: 429, 1952.

⁴Dresbach, M.: *Science* 21: 477, 1904.

⁵Bishop, F. W.: *Arch. Int. Med.* 14: 388, 1914.

Sydenstricker³ reported a case of a Negro with 90 per cent of his red cells oval. Lawrence⁴ reported a case of a white woman, 32 years of age, with many oval red blood cells. Bernhardt⁵ described the case of a white woman, 34 years of age, with oval red cells. Bernhardt termed this condition ovalocytosis. Miller and Lucas⁶ have reported nine additional cases, in four generations.

It must be understood that elliptical erythrocytes are not poikilocytes of any of the known anemias, including sickle cell anemia. It is a congenital anomaly compatible with an otherwise normal individual. It must be recalled that all mammals except the terrestrial family *Camelidae* show circular erythrocytes. Conversely, among the lower vertebrates, the red blood cells are elliptical with the single exception of the marine family *Cyclostomata*. Dresbach in 1902 first reported elliptical red blood cells in human beings. In the subsequent cases reported, it was found that males and females are equally affected. It has been observed in a number of different nationalities. Its familial tendency was proved by Hunter and Adams⁷ in 1929.

As stated above the etiology is unknown. It has been laid at the door of faulty erythropoiesis, influences of the blood plasma, the inherent quality of the cells, racial characteristics, anoxemia, and it has been suggested that the etiology is similar to that of sickle cell anemia. Normal erythrocytes are unaltered by the plasma of persons with this anomaly. By means of autopsy, bone marrow and splenic punctures, it has been shown that these cells are not elliptical when they are formed in the bone marrow, but assume that shape when subjected to the influence of some unknown constituent of the plasma or extramarrow tissues. That normal erythrocytes are unaffected by the plasma from a case of ovalocytosis suggests a second intraerythrocytic susceptibility to the extraerythrocytic influence, either or both of which may be congenital.

Miller and Lucas comment upon the fact that mechanical effects, temperature, normal blood serum, iso- and hypotonic saline washing, picric acid, carbon dioxide, oxygen, cholesterol, etc., all failed to affect the constancy of the ellipsoid state of the red cells. Terry has shown that elliptical erythrocytes are more resistant to hemolytic agents and have a decreased fragility. He⁸ has shown that these cells are heavier than round cells. Pollock and Dameshek⁹ observed that potassium cyanide increased the number of elliptical erythrocytes very rapidly.

The medicolegal point about this phenomenon is interesting; namely, within certain limits it may be used as a means of identification of both persons and paternity.

The cases reported by Miller and Lucas occurred in four generations. Twenty relatives were studied and none showed these elliptical cells. There were in this family five males and four females, varying in age from 30 days to 84 years, who had elliptical erythrocytes.

³Sydenstricker, V. P.: J. A. M. A. 81: 133, 1923.

⁴Lawrence, J. S.: J. Clin. Invest. 5: 31, 1927.

⁵Bernhardt, H.: Deutsch. med. Wchnschr. 54: 987, 1928.

⁶Miller and Lucas: Am. J. Clin. Path. 8: 391-397, 1938.

⁷Hunter and Adams: Ann. Int. Med. 2: 1162, 1929.

⁸Terry, M. C., et al.: Arch. Path. 13: 193, 1932.

⁹Pollock and Dameshek: Am. J. M. Sc. 188: 822, 1934.

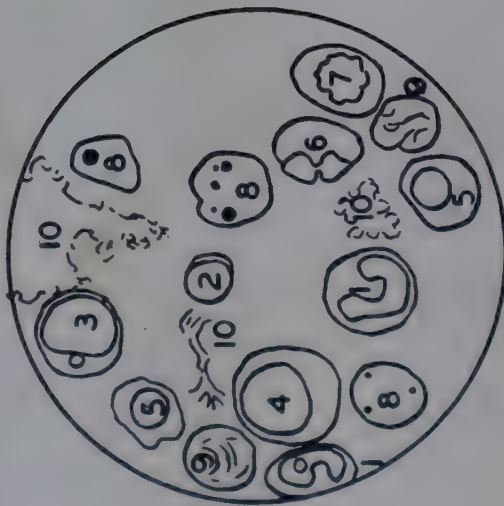
SICKLE CELL ANEMIA



BONE MARROW

1. Normoblasts in karyorrhexis
2. Polychromatic normoblast
3. Orthochromatic normoblast with three nuclei
4. Fat
5. "Stab"
6. Neutrophilic juvenile
7. Neutrophilic myelocyte
8. Segmented neutrophile
9. Myeloblast
10. Polychromatic megakaryoblast
11. Lymphocytes (?)
12. Nuclei of normoblasts
13. Immature nuclear structure

SICKLE CELL ANEMIA

BLOOD FILM
REGENERATION

1. "Stab"
2. Lymphocyte
3. Neutrophilic myelocyte
4. Orthochromatic megakaryoblast
5. Orthochromatic macroblast
6. Polychromatic macroblast with karyorrhexis
7. Orthochromatic macroblast with basophilic punctation and karyorrhexis
8. Howell-Jolly bodies
9. Polychromatic macrophocyte with endoglobular degeneration
10. Groups of blood platelets
11. Artefact

SICKLE CELL ANEMIA



BLOOD FILM
REGENERATION



WET PREPARATION

PLATE XVII.



BONE MARROW

Since the above article was written there has appeared another family of three generations in which ten members exhibited elliptical cells. The report was made by Strauss and Daland.⁴

Motulsky and associates⁵ divide patients with hereditary elliptocytosis into four groups: (I) normal, round erythrocytes, (II) oval erythrocytes, (III) elliptical erythrocytes, and (IV) rod or cigar-shaped erythrocytes. They state that the predominance of group IV cells favors a diagnosis of hereditary ovalocytosis, whereas an association of elliptical cells and other deformed cells is more indicative of symptomatic ovalocytosis such as occurs with pernicious anemia, Cooley's anemia, severe iron deficiency, leukemia, cancer, and severe anemia associated with infection. In one of three cases presented, the survival time of transfused red cells was found reduced to 45 days. This case presented other evidence of hemolysis. On the other hand, the patients with normal survival times showed no hemolytic features. These authors call attention to the similarity of hereditary ovalocytosis with other diseases characterized by deformity of the red cell and shortened life span of these cells, such as occur in sickle cell anemia, hereditary spherocytosis, and thalassemia major. Just as in these syndromes, where there are a "disease" group and a nonsymptomatic "trait" group, ovalocytosis may likewise be subdivided. Red cell survival times in the trait group are normal, whereas in the disease group they are shortened. This survival time difference in the two groups is not dependent on elliptocytes; the cause for such differences remains unknown. Molecular disease of hemoglobin accounts for the changes in sickle cell, thalassemia, and other diseases, but electrophoretic studies in two of these three cases were normal and fetal hemoglobin was not increased. Motulsky and associates review the literature on symptomatology and heredity patterns in the hemolytic syndromes associated with erythrocyte shape anomaly and show the many points of resemblance between elliptocytosis and the other hemolytic groups. The hereditary pattern is considered a Mendelian dominant. Splenectomy is advised in the severe condition but not in the nonhemolytic or "compensated" form of the disease.

Target Cell Anemia

The term "target cell" was used by A. M. Barrett⁶ in 1938. These peculiar erythrocytes were described in a condition called "target cell anemia." This type of red cell, while described by Barrett, was really first alluded to as a special type of erythrocyte by Haden and Evans in 1937.⁷ They called them "dimpled corpuscles." Haden and Evans believed they were present only in sickle cell anemia but Barrett maintained that they were to be found in some cases of obstructive jaundice and severe hepatitis, in some cases of hypochromic anemia, and following splenectomy. In addition to this, Dameshek,⁸ described a case of target cell anemia, which corresponded to what he termed an "anerythroblastic type of Cooley's erythroblastic anemia."

⁴Strauss, M. B., and Daland, G. A.: Hereditary Ovalocytosis: Observations on Ten Cases in One Family, *New England J. Med.* 217: 100-103, 1937.

⁵Motulsky, A. G., Singer, K., Crosby, W. H., and Smith, V.: *Blood* 9: 57, 1954.

⁶Barrett: *A. M. J. Path. and Bacteriol.* 46: 604, 1938.

⁷*Arch. Int. Med.*, 1937.

⁸Dameshek, W.: *Am. J. M. Sc.* 200: 445, 1940.

The description of this cell, according to Dameshek, is about as follows: the cell is abnormally thin, tends to "buckle" and to present in stained preparations a bull's eye appearance. In contradistinction to the thick spherocyte, the target cells are unusually resistant to hypotonic solutions of sodium chloride. When these cells are present in any significant number (over 4 per cent) they result in a lengthening of the fragility span, with complete hemolysis occurring between 0.16 and 0.02 per cent, depending upon the number of these cells present. In the cases described, hemolysis was incomplete at a concentration of sodium chloride of 0.04 per cent. He found that by examination of the centrifuged red cells from concentrations of sodium chloride below 0.30 per cent, following resuspension in the patient's own (heparinized) plasma, the high concentration of target cells was demonstrated. When the red cells were suspended in varying concentrations of hypotonic solutions of sodium chloride and the hematocrits determined, they found in a concentration of 0.30, 45 per cent hematocrit, with mean corpuscular volume of 144. The target cell is the antithesis of the spherocyte.

Target cell anemia may be closely related to or indeed even a phase of the condition known as erythroblastic or Mediterranean anemia.

Dameshek for the first time reported a type of target cell anemia which he classified as *forme fruste* or an anerythroblastic type of Cooley's anemia or a closely related condition. This was an Italian youth with hypochromic anemia, splenomegaly, and a hemolytic type of icterus. About one-third of his red blood cells presented the appearance of "targets" or "bull's eyes"; these cells were unusually resistant to hypotonic solutions of sodium chloride. No nucleated red cells were present. Generalized osteoporotic changes with great thickening of the skull were present; in addition a bony tumor arising from a rib encroached on the right upper lung. Dameshek suggested the possibility that Cooley's anemia, sickle cell anemia, and target cell anemia are related conditions with target cells and increased saline resistance as common denominators. In Cooley's anemia, the target cell is probably a more fundamental abnormality than the erythroblast and may represent the basic hereditary defect. In these atypical hemolytic states, a lack of response to splenectomy further differentiates them from most cases of congenital and acquired hemolytic icterus.

Bohrod¹ believes that the target cell is a newly formed cell produced by the bone marrow in response to blood loss regardless of the cause. In support of this argument, he states that the appearance of target cells in the stained blood film, far from being an unusual event, is very common. Such cells can be found in small numbers, up to 1 per cent or 2 per cent, early in the majority of cases of anemia. Rare ones may even sometimes be seen in apparently normal spreads. In more chronic anemias the number is variable.

The presence of the target cells in stained spreads is transitory in acute anemia. They often disappear before there is a significant rise of the red cell count, in this way resembling the reticulocyte. In several cases they have been present for only one or two days. They usually first appear on the third or fourth day following a hemorrhage, although in one case they were first

¹Bohrod, M. G.: *Am. J. M. Sc.* 202: 869, 1941.

seen on the day after a hemorrhage from peptic ulcer. Coincident with their appearance there is a transitory decreased fragility of the erythrocytes. Bohrod cites the following cases:

Case Abstract.—

Case 1.—A woman, aged 23, lost considerable blood during and following the delivery of her second child. On the second postpartum day her erythrocyte count was 3,400,000, her hemoglobin 67% Sahli, color index 0.98. There were no target cells; the stained films appeared normal. The next day a few polychromatophilic erythrocytes were present and some of these were target cells. On the fourth and fifth postpartum days polychromatophilic cells were more numerous (between 1% and 2%) and nearly every one was a target cell. None of the normally stained cells were target cells, and a few target forms were seen in acidophilic cells. The important point is that *at one stage only those cells showed the target forms which by their polychromatophilia indicated their recent formation in response to the blood loss.* By the time of the patient's discharge from the hospital on the twelfth postpartum day the erythrocyte count was 4,100,000, hemoglobin 76%, color index 0.92. The stained film appeared normal; there were no polychromatophilic cells and no target cells.

In most cases target cells are seen only after a moderately severe hemorrhage. The loss of blood may be slight, as illustrated by the following case cited by Bohrod:

Case 2.—A woman received a laceration of the scalp in an automobile accident. Bleeding was, according to her, profuse, but it was stopped in about 45 minutes. No blood count was made until the fourth day later, when it was found to be 4,500,000; 2% of the erythrocytes were target cells. Three days later target cells had disappeared although the erythrocyte count had not changed.

Target cells are of normal diameter. In the stained spread they are made up of a rim of hemoglobin-containing area, with two areas being separated by a zone which is clear and unstained. Barrett believed that this appearance was due to a central knoblike projection in a cup or bowl-shaped cell and showed that a body of such shape would look like a target when viewed on the broad surface. That this is actually the shape of the cell can be seen by oblique illumination of dry films or in bas-relief photographs. No target cells are seen in wet preparations. Instead, a number of cells, equivalent to the proportion which appear as target cells in the stained film, appear as shallow or, more uncommonly, deep bowls or cups. When blood is diluted with hypotonic salt solutions of varying concentrations, the proportion of bowl-shaped cells, or of target cells in dry films, increases as the salt concentration decreases. This is due to the hemolysis of the normal-appearing cells and the preservation of the target cells.

Bohrod does not believe that the target cell is a fundamentally deficient cell in any type of anemia. He believes that the target cell is merely a hyper-resistant young cell which appears in the blood stream following loss of blood. He believes that in acute anemias they are present for only a short time early in the regenerative phase, and they have disappeared by the time a significant rise of erythrocyte count is evident. Also, he is convinced that the target cell is a hyperresistant cell produced by the bone marrow in response to the blood loss. Increased resistance to the hemolytic action of hypotonic saline and to acetic acid has been demonstrated.

Bohrod's views differ widely from those of Dameshek in that he does not believe that the contention that the target cell represents the fundamental

defect in Cooley's anemia is justified. It is simpler, in Bohrod's opinion, in light of available evidence, to regard the target cell in this disease, as in sickle cell anemia and in many other anemias, as a response to the blood destruction rather than as the cause of it.

Since the last edition of this text, a great deal of work has been done in diseases associated with the target cells. Much of this material is covered in the section on "Molecular Disease of Hemoglobin," pages 734 ff. *Thalassemia major* has been designated the symptomatic form, with marked anemia, hemolytic crises, and numerous target cells (see page 731). In this condition, Singer and his co-workers have demonstrated high fetal hemoglobin values ranging from 40 to 100 per cent. The trait state, *thalassemia minor*, may be found in members of the family of patients with the disease. These individuals are asymptomatic but have target cells in small numbers in their blood. Fetal hemoglobin in the trait group may be increased up to 10 per cent. Since these original publications, *thalassemia* has been described in conjunction with other molecular diseases of hemoglobin. These combinations include sickle-thalassemia disease, *thalassemia-hemoglobin C* disease, and *thalassemia-hemoglobin E* disease.

The mode of inheritance of *thalassemia*¹ reveals that both parents of such patients are heterozygous, but a homozygous combination of the pathologic genes results in offspring displaying severe hemolytic disease. The heterozygous group usually have no hemolytic phenomena and a normal red cell survival time, as compared with a shortened survival time in the homozygous state. Eadie and Brown² believe that the shortened life span is due to random destruction of cells.

Aplastic Anemia

Aplastic anemia is usually regarded as a well-defined, readily recognizable clinical entity, with features that are the result of a progressive depression and final cessation of bone marrow activity and aplasia of this tissue. Ordinarily, one does not think of regeneration in these cases, but Thompson, Richter and Edsall³ reported thirteen cases of aplastic anemia in which there was evidence of blood regeneration. The first case of aplastic anemia on record is that by Ehrlich.⁴ In this case the red count was 213,000, no white count reported, no blood platelets reported, tibial marrow yellow and fatty. By 1908 Cabot⁵ collected 24 cases and Hirschfeld,⁶ in 1911, found the total to be 44. Musser⁷ reviewed the situation up to 1914. In 1919, Smith collected 64 cases. He found that it was a disease of young adults that progressed rapidly and it was characterized by a progressive decrease in the number of formed elements in the blood. Root⁸ and Carey and Taylor collected, in 1931, 150 cases.

¹Valentine, W. N., and Neel, J. V.: *Arch. Int. Med.* 74: 185, 1944.

²Eadie, G. S., and Brown, I. W., Jr.: *Blood* 8: 1110, 1953.

³Thompson, W. P., Richter, M. N., and Edsall, K. S.: *Am. J. M. Sc.* 187: 77, 1934.

⁴Ehrlich, P.: *Charité Ann.* 18: 300, 1880.

⁵Cabot, R.: *Osler's System of Med.* 4: 637, ed. 1, 1908.

⁶Hirschfeld: *Folia haematol.* 12: 347, 1911.

⁷Musser, J. H.: *Arch. Int. Med.* 14: 275, 1914.

⁸Root, J. H.: *New England J. Med.* 203: 1925, 1930

It is accepted that idiopathic aplastic anemia is a uniformly acute, rapidly fatal disease of adolescents and young adults, with a high fever, a rapid decrease in the cells of the blood with very little suggestion of regeneration of the blood cells. At autopsy, the bone marrow shows complete fatty degeneration.

The blood picture is a lowered count, often below 2,000,000; a marked lowering of hemoglobin with the color index usually below 1.0. While there is a rapid decline in the number of red cells, the form of the cells is not markedly changed. It is significant that there are no nucleated red cells and very little polychromasia. Reticulocytes are very scarce. The white cell count declines with a decline of the neutrophils and disappearance of eosinophiles, and a relative lymphocytosis supervenes. There is a marked leukopenia in advanced cases of this disease. The final picture, so far as leukocytes are concerned, is practically all lymphocytes. Another very characteristic phenomenon in the blood picture is the decline and the absence of blood platelets. It is significant that when the blood platelets drop very low, purpuric symptoms occur.

From a study of the literature, it seems that there are three variants of aplastic anemia. There are cases in which the disease is acute in its course, without evidence of regeneration, and with a complete aplasia of the marrow at autopsy. In addition, there is a second group comprising a few cases with clinical features similar to the preceding group, but in which the marrow, instead of being fatty, was found to have a normal or even increased cellular content. Such cases have been reported by Naegeli,² Senator,³ Wolff,⁴ and Root⁵ and several appear in the Italian literature⁶⁻⁸ under the name of "pseudoplastic anemia," a term suggested by Luzzatto⁸ to indicate the discrepancy between the blood and bone marrow picture. The third variation includes progressive hypocythemia cases in which evidence of regeneration appeared in the peripheral blood.

According to Thompson, Richter, and Edsall, examination of the peripheral blood may be no index as to the morphologic appearance of bone marrow examined, and the morphologic appearance of the bone marrow may be no index as to its functional activity. There may be quite serious interference with the normal course of development, maturation, and division of blood cells without alteration in the cellular content of the marrow.

Krumbhaar in commenting upon the case reports and conclusions of Thompson, Richter, and Edsall, stated very properly that these conditions should be described by three different labels as follows: "aplastic anemia" is a term to be reserved for the type without evidence of regeneration in blood or bone marrow; "pseudoaplastic anemia" for the variety with cellular bone marrow; and "progressive hypocythemia" for the cases corresponding to those of Thompson, Richter, and Edsall where there is an evidence of attempted regeneration in both bone marrow and blood.

²Naegeli, O.: *Blut-Krankheiten und Blut-Diagnostik*, Berlin, 1931, Julius Springer.

³Senator, H.: *Ztschr. f. klin. Med.* 54: 1, 1904.

⁴Wolff, A.: *Berl. klin. Wchnschr.* 42: 35, 1905.

⁵Root, J. H.: *New England J. Med.* 203: 1925, 1930.

⁶DiGuglielmo, G.: *Boll. della Soc. med. chir. di Pavia* 3: 131, 1928.

⁷Introzzi, P.: *Hematologica* 7: 35, 1926.

⁸Luzzatto, A. M.: *Riv. ven.* 47: 193, 1905.

Hookworm Anemia

Hookworm anemia has been known since the construction of the Gotthard tunnel in Switzerland in the years 1876-1880. Perroncito¹ noted that many Italian workers developed a severe and at times fatal anemia, with hookworm infestation. Later, hookworm anemia was called miner's anemia because of its occurrence in mines. Boycott² described cases of hookworm anemia in England (1901). Later hookworm anemia was observed in other European countries, in America, and in tropical countries, where suitable conditions for hookworm infestation exist.

The etiology of this disease has been well discussed in many papers. Stransky and Quintos³ gave an accurate description of it from their experience in the Philippines, where the *Ancylostoma* is very common.

They observed three types or stages of blood changes in hookworm disease. In the first type the bone marrow is able to maintain the equilibrium between blood loss and production, and there is no manifest anemia. They call this the stage of "compensated anemia." In the second type the production is unable to counterbalance the blood loss, and a hypochromic anemia results. In the third type the bone marrow is exhausted, and aplastic anemia develops. In the first type they found in the peripheral blood eosinophilia and reticulocytosis without anemia. The bone marrow findings were characterized by increased erythropoietic activity and likewise eosinophilia. Despite a continuous loss of blood, an increased production in the bone marrow was sufficient to maintain the equilibrium between blood formation and blood loss. In the early stage of hookworm disease, real anemia may not develop if food and living conditions are adequate.

In the second type, they described these characteristics—anemia, microcytosis, hypochromia, eosinophilia, and reticulocytosis in the peripheral blood; and eosinophilia and marked erythropoietic activity of the bone marrow.

Hookworm anemia is hypochromic and microcytic, with a red cell count of 1.0 to 3.5 millions per cubic millimeter. In advanced cases the red cell count may drop to below one million. Eosinophilia usually amounts to from 5 to 15 per cent, although in more advanced cases eosinophilia may no longer be present. In some cases there may be a hypoplastic tendency of the bone marrow, but the condition is still reversible and the patient recovers.

The third type, or irreversible type (aplastic anemia), is characterized clinically by severe anemia, dizziness, easy fatigability, and hemorrhagic diathesis. The erythropoietic activity of the bone marrow is almost nil. There is so-called panmyelophthisis with low cell count; relative lymphocytosis; and lack of erythropoietic, granulocytopoietic, and thrombocytopoietic activity of the bone marrow. In this stage antihelminthic and antianemic treatments are of no avail. Blood transfusions and iron are ineffective in improving the irreversible process. Aplastic anemia as a possible outcome of hookworm infestation has only rarely been mentioned. Stransky and Quintos observed eight cases of aplastic anemia in hookworm disease. They observed during the same time five so-called primary idiopathic aplastic anemias. In one case there were

¹Perroncito, L.: *Ann. d. real. Accad. di Agricoltura di Torino*. 22: 219, 1880.

²Boycott, A. E.: *Ankylostomiasis*, tr. *Epidemiol. Soc.*, London 24: 113, 1904-1905.

³Stransky, E., and Quintos, F. N.: *Blood* 2: 1, 63-71, Jan., 1947.

hypochromia, anemia, leukopenia with granulocytopenia, and relative lymphocytosis, thrombocytopenia, and reticulocytopenia in the peripheral blood. There were low cell count, relative lymphocytosis, poor erythropoietic activity, and megakaryocytopenia in the bone marrow. In two other cases, while the outcome was the same as aplastic anemia, the hematologic picture at first was not definitely that of panmyelophthisis. One of these cases was an example of severe anemia with symptoms of early hemorrhagic diathesis. From the hematologic picture alone, the diagnosis of aplastic anemia could not be definitely made during the stay of the patient in the hospital for seven weeks. However, the outcome was fatal, and in two weeks after discharge, there was probably a rapid breakdown of the bone marrow. In another case, anemia was not extreme at first, with a count above one million; bone marrow count was 105,000, which is about normal, and the differential count was not much altered; but the erythropoietic activity of the bone marrow was low, the ratio of nucleated red cells to white cells was 0.19:1.0 compared to 0.3:1.0; 0.6:1.0; and 1.1:1.0 in those that recovered in the second type. The hemorrhagic symptoms, leukopenia with relative lymphocytosis, thrombocytopenia, low reticulocyte count, and absence of nucleated red cells in spite of the anemia, shown by the peripheral blood, were in favor of aplastic anemia even in the beginning of the observation, despite the somewhat normal bone marrow findings. They believed that their cases of aplastic anemia were not simply coincidental with hookworm anemia but were actually related to it. They believed that the aplastic anemia of hookworm disease is due to bone marrow exhaustion following continuous blood loss in the presence of various factors, including dietary deficiency.

Hematology of Lead Poisoning

The effects of alimentary, respiratory, or cutaneous contact of lead with the human body lead to a definite change in the microscopic picture of the red blood cells; in the main, the appearance of the classical basophilic punctation and a mild degree of polychromasia. These changes in the blood are the first and most striking signs of lead poisoning in man. Hematology is indebted to the work of Aub, Reznikoff, and Smith¹ for definite information on just how lead acts specifically upon the blood system. Their experiments in vitro with blood and lead demonstrate two sets of facts: first, the physiologic, physical, and chemical reactions of lead on isolated cells, indicating a similar action on other body cells; and second, the production of at least one type of secondary anemia. The experimental researches by Aub, Reznikoff, and Smith, and P. Schmidt² serve to guide one in understanding the effects of lead upon the human system, although the complete story has not by any means been written.

According to Aub and others, Laennec³ first recognized lead anemia in man. Exposure of experimental animals to lead is followed by marked anemia. This was demonstrated in rabbits by intramuscular injection by Carcanaque and Maurel⁴ and after oral administration by Key.⁵

¹Aub, J. C., Reznikoff, P., and Smith, D. E.: *J. Exper. Med.* **40**: 151, 1924.

²Schmidt, P., Seiser and Litzner: *Ergebnisse der ges. Medizin*, Bd. XIII, 1929.

³Laennec, R. T. H.: *Traite de l'auscultation meditee*, Paris, ed. 3, 1831.

⁴Carcanaque and Maurel: *Compt. rend. Soc. de biol.* **74**: 377, 1913.

⁵Key, J. A.: *Am. J. Phys.* **70**: No. 1, 86-97 (Sept.), 1924.

Two causes of this anemia have been described: marked destruction of blood, or a lesion in the bone marrow with consequent deficient blood formation. According to Stockman and Charteris,¹ and Raimondi,² bone marrow of rabbits showed a marked preliminary increase of leukoblasts with disappearance of fat, followed later by definite gelatinous degeneration. Wolff³ described a case of severe lead poisoning in which attempts to regenerate blood were followed by deficient hematopoietic function, as seen in the bone marrow. Meillère,⁴ Schnitter,⁵ and Sellers⁶ thought that lead definitely injured both blood cells and bone marrow. Bouchard,⁷ and later Rauch⁸ and others, attributed the primary appearance of anemia to abnormal destruction of the cells in the circulation, even though degeneration of the bone marrow may occur later. Evidence for this is diverse. Early lead poisoning shows hyperplasia of the bone marrow⁹ with many nucleated red cells in the circulation which seem to indicate regeneration. Key¹⁰ demonstrated that within 24 hours after ingestion of 1 gm. of lead the number of circulating erythrocytes in rabbits decreased more than 20 per cent, with the appearance of many nucleated red cells. Meillère¹¹ showed a definite increase in urinary excretion of iron, largely derived from erythrocytes, in the onset of lead anemia. J. W. S. Brady,¹² in Aub's laboratory, demonstrated that slow intravenous injection of lead acetate dissolved in saline caused marked hematuria and very marked increase in excretion of bile pigments from the common duct. These unpublished experiments on rabbits indicate definitely and very clearly that small quantities of lead in the blood stream cause great destruction of blood, not accounted for by any osmotic changes. Chester Jones¹³ obtained similar evidence in human cases. He showed that both the blood plasma and bile obtained by duodenal drainage from several cases of plumbism contained very high concentrations of bile pigments. These facts indicate marked peripheral destruction of blood due to ingestion of lead. No good explanation of this increased blood destruction has been previously suggested because of the lack of thorough experimentation upon the subject.

Observations of several phenomena associated with the blood in lead poisoning have been made. Behrend¹⁴ in 1899 first observed the stippling of erythrocytes in lead poisoning. Key¹⁵ in 1922 summarized the many reports appearing in the literature since Behrend's observation. It is probable that stippled erythrocytes are really young red cells degenerating as a result of exposure to lead. Since stippling cannot be produced in vitro¹⁶ the mechanism of their development is not yet understood.

¹Stockman, R., and Charteris, F. J.: *J. Path. & Bact.* 9: 202, 1904.

²Raimondi, C.: *Ann. Univ. Med. e Chir.* 251: 52, 1880.

³Wolff, A.: *Berl. klin. Wehnschr.* 39: 840, 1902.

⁴Meillère, G.: *Le saturnisme; étude historique, physiologique, clinique, et prophylactique.* Paris, 1903.

⁵Schnitter: *Deutsch. Arch. klin. Med.* 117: 127, 1915.

⁶Sellers, A.: *J. Ind. Hyg.* 2: 361, 1920-21.

⁷Bouchard: *Compt. rend. Soc. de biol.*, v, series 5, 358, 1873.

⁸Rauch, H.: *Ges. exper. Med.* 28: 50, 1922.

⁹Stockman, R., and Charteris, F. J.: *J. Path. & Bact.* 9: 202, 1904.

¹⁰Key, J. A.: *Am. J. Physiol.* 70: No. 1, 1924.

¹¹Meillère, G.: *Le saturnisme; étude historique, physiologique, clinique et prophylactique.* Paris, 1903.

¹²Brady, J. W. S., unpublished data.

¹³Jones, C., unpublished data.

¹⁴Behrend: *Deutsch. med. Wehnschr.* 25: 254, 1899.

¹⁵Key, J. A.: *loc. cit.*

¹⁶Pepper, O. H. P.: *Arch. Int. Med.* 30: 801, 1922.

Erythrocytes in lead poisoning show increased resistance to hemolysis in dilute saline solution. Malassez¹⁴ reported in 1873 that not only is anemia a symptom of lead poisoning, but that the erythrocytes are larger and more fixed than normal. Agasse-Lafont and Heim¹⁵ found slightly increased globular resistance in lead poisoning. Von Liebermann,¹⁶ and later Orban¹⁷ and Hayhurst¹⁸ demonstrated this. Fici¹⁹ studied this reaction in vitro in 1921 and found that lead acetate exerts a striking effect on the hemolysis of red cells in hypotonic saline solutions, varying with concentration of lead and with temperature.

Fici's observations have been confirmed by the work of Aub, Reznikoff, and Smith. It appears, from the investigations of other laboratories, that the anemia observed in cases of lead poisoning is due to *destruction of blood* rather than to *diminished production of blood*. The method of poisoning cells in vitro with lead was adopted by Aub and his coworkers in order to study this phenomenon, and distinct effects were thereby obtained, even when only 0.001 mg. of lead is added to approximately 5 billion washed red corpuscles. In order to obtain optimum results the usual dosage employed was ten times this or 0.01 mg. per 5 billion cells. After this exposure, they noted the following changes in the cells treated: such a marked increase in the resistance to hypotonic salt solution developed that complete hemolysis did not occur until the cells were exposed to a saline solution of 0.05 per cent. Untreated cells were completely hemolyzed in 0.25 or 0.225 per cent saline. This reaction was quantitative and varied with the concentration of lead used. Under the conditions of the experiments this phenomenon seemed to be unique. The effects of arsenic were very slight in comparison. While from this reaction it may be concluded that lead increased cellular resistance, it also appeared that it shortened the life of blood cells. This may be demonstrated by the much more rapid appearance of hemolysis than normal when the cells were merely allowed to stand in Ringer solution of any dilution. In rabbits with acute lead poisoning these same phenomena were noted in vivo. Both phenomena may be changed in vitro by varying the time and temperature of the reaction and the concentration of lead, as Fici has already pointed out. If normal cells stood in Ringer solution for six hours, something diffused into the solution which largely reduced the action of lead. After repeated washing these cells reacted with lead in the usual manner. Small amounts of serum reacted with lead and eliminated its effects. Red blood cells, treated with a mixture of lead and blood serum, showed normal hemolysis in hypotonic salt solution. When lead was added to whole blood, the only evidence of this neutralization was a marked decrease in the intensity of the reaction. This is probably explained by the fact that since both serum and cells were present together, the lead reacted with both simultaneously. This in turn explains how the reaction may occur in vivo. The change in hemolysis

¹⁴Malassez, L.: Compt. rend. Soc. de biol. v, series 5, 125, 1873.

¹⁵Agasse-Lafont, E., and Heim, G.: Brussels, September, 1910.

¹⁶Von Liebermann, L., and von Fillinger, F.: Deutsch. med. Wchnschr. 38: 2079; 1912.

¹⁷Orban, R.: Deutsch. med. Wchnschr. 38: 2079, 1912.

¹⁸Hayhurst, E. R.: Survey—under Board of Health, Columbus, 1915.

¹⁹Fici, V.: Folia Med. 6: 587, 1920; 7: 82, 201, 1921.

does not appear in the blood of all species of animals. In the species in which it does occur, anemia and stippling of the red cells also tended to develop readily during lead intoxication. In vitro, cells which have been exposed to lead were far more fragile than normal blood cells. Slight trauma caused them to hemolyze. In vivo this was probably an important causal factor in the increased destruction of blood and in the anemia of acute lead poisoning. The serum of a rabbit, which has been immunized to human blood, caused more rapid and complete hemolysis in cells treated with lead than in the control cells. No satisfactory explanation for this has been found.

Further studies by Aub, Reznikoff, and Smith¹ on the effects of lead on red blood cells so far as surface phenomena and their physiologic explanation were concerned, showed the following: partial loss of the normal stickiness of red corpuscles, which was demonstrated by their falling from a clean glass surface; loss of the agglutination reaction which normally followed mixture with serum of a different isoagglutinating group; decrease in volume even in isotonic solutions; loss of normal elasticity and, therefore, reduced changes in volume upon exposure to marked variations in osmotic tension; increase in resistance to large changes in external osmotic pressure because of this inelasticity, and therefore decreased hemolysis in hypotonic salt solution; increase in the speed of disintegration in spite of this increased resistance to external osmotic pressure. "Leaded" cells broke up more readily upon standing than did normal cells, and were easily fractured by rotation or shaking.

All these phenomena seemed to be associated largely with surface changes in the corpuscles. Evidence was cited that there was no chemical reaction between lead and hemoglobin. The gas exchange was identical in normal and "leaded" cells. The function of the interior of the red cells, therefore, appeared to be unaffected by lead.

The effects of lead upon red blood cells were thus manifested by shrinkage, inability to expand, increased brittleness, and loss of the normal consistency which made their surface sticky. After exposure to lead, red blood corpuscles were more like hard inelastic brittle rubber balls, than like the soft, elastic, resilient cells characteristic of normal blood.

A third communication by Aub and Reznikoff* gave a very comprehensive chemical explanation of the reaction of lead with red blood cells. They showed that the changes in red blood cells caused by lead were explained on the basis of the union of lead with inorganic phosphate and were largely surface phenomena. It was found by them that lecithin, cholesterol, euglobulin, pseudoglobulin, hemoglobin, and albumin do not interfere with the reaction. Sodium bicarbonate can neutralize lead; but ten times as much is required as occurs in an equivalent neutralizing quantity of serum. This carbonate can therefore play only a small role in the reaction. They found that inorganic phosphate in the same concentration as is present normally in serum neutralizes the same quantity of lead as does the whole serum itself. This can, therefore, completely account for the action of lead on red blood

*J. Exper. Med. 40: 189, 1924.

¹Aub, J. C., Reznikoff, P., and Smith, D. E.: J. Exper. Med. 40: 186, 1924.

cells. Adding inorganic phosphate to red blood cells or to whole blood greatly decreases the action of lead on the cells but cannot eliminate it completely. This indicates that there is a simultaneous reaction between lead and serum and lead and corpuscles when all are present, and explains the action of lead in vivo. Serum with increased inorganic phosphate content, obtained from two patients, neutralized the effect of lead to a high degree. In one case serum with a high normal phosphorus content had a similar though less marked action.

They concluded further that diffusion of inorganic phosphate from erythrocytes into the surrounding Ringer solution prevents the action of lead upon the corpuscles, due to neutralization of the lead by the diffusate. If the diffusate is washed from these same cells, lead will again react with them. These findings indicate that the union of lead with the inorganic phosphate of the cells explains the action of lead on erythrocytes. Interaction of lead salts and phosphate results in the formation of a very insoluble lead phosphate $[Pb_3(PO_4)_2]$ and free acid (Fairhall). When a collodion membrane separates solutions of lead chloride and phosphate or serum, lead phosphate is precipitated in the membrane with a local formation of acid. This acid, formed as the result of the union of lead and phosphate, may perhaps change the physicochemical state of the colloids of the cell surface. Macroscopic cataphoresis determinations demonstrated no difference between migration of "leaded" and normal cells. Agglutination experiments with colloidal iron and arsenic showed a marked difference between "leaded" and normal cells. All this points to further evidence of changes in cell surface.

The three papers by Aub and Reznikoff suffice to explain the anemia of lead poisoning. Red cells in vitro showed various changes after exposure to a very small amount of lead. The surface of the cells is changed. Their permeability to water is so altered that they shrink and are incapable of swelling as much as normal cells. This is associated with a marked increase in resistance to different osmotic surroundings. They show less hemolysis than normal in very weak salt solutions. "Leaded" cells hemolyze readily as a result of slight trauma and are very short lived. These cells also lose their normal stickiness and are no longer agglutinated by sera of incompatible isoagglutinating groups. These changes are evidences of an effect on the surface of the cell, not the interior, which does not undergo disturbances, at least insofar as the physiologic properties of the hemoglobin are concerned. These physical changes are brought about by a precipitation of insoluble lead phosphate and a formation of acid, causing the "leaded" erythrocyte to change from an elastic distensible sac to one which is contracted, relatively inelastic and brittle. This altered cell cannot withstand the trauma involved in the circulation of the blood, which probably explains the marked destruction of peripheral blood in lead poisoning.

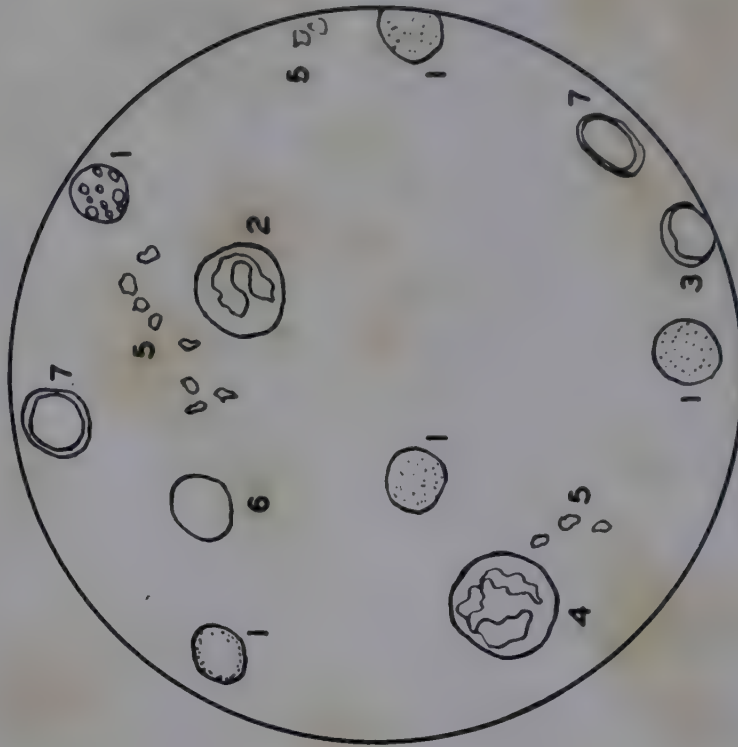
It is believed that basophilic punctation is an effect of lead, but the presence of basophilic punctation alone does not prove the case. One must have history, clinical symptoms such as lead colic, the lead line on the gums, the finding of lead in the urine, etc.; all these serve to clinch the picture. P. Schmidt has well said that basophilic punctation is a "valuable aid before the appear-

HYPOCHROMIC ANEMIA



1. Blood platelet on an erythrocyte
2. Group of blood platelets
3. Polychromatic normoblast
4. Large lymphocyte with azurophilic stippling
5. Polychromatic erythrocyte
6. Basophilic punctated erythrocyte
7. Monocyte
8. Erythrocyte showing hypochromia

LEAD POISONING

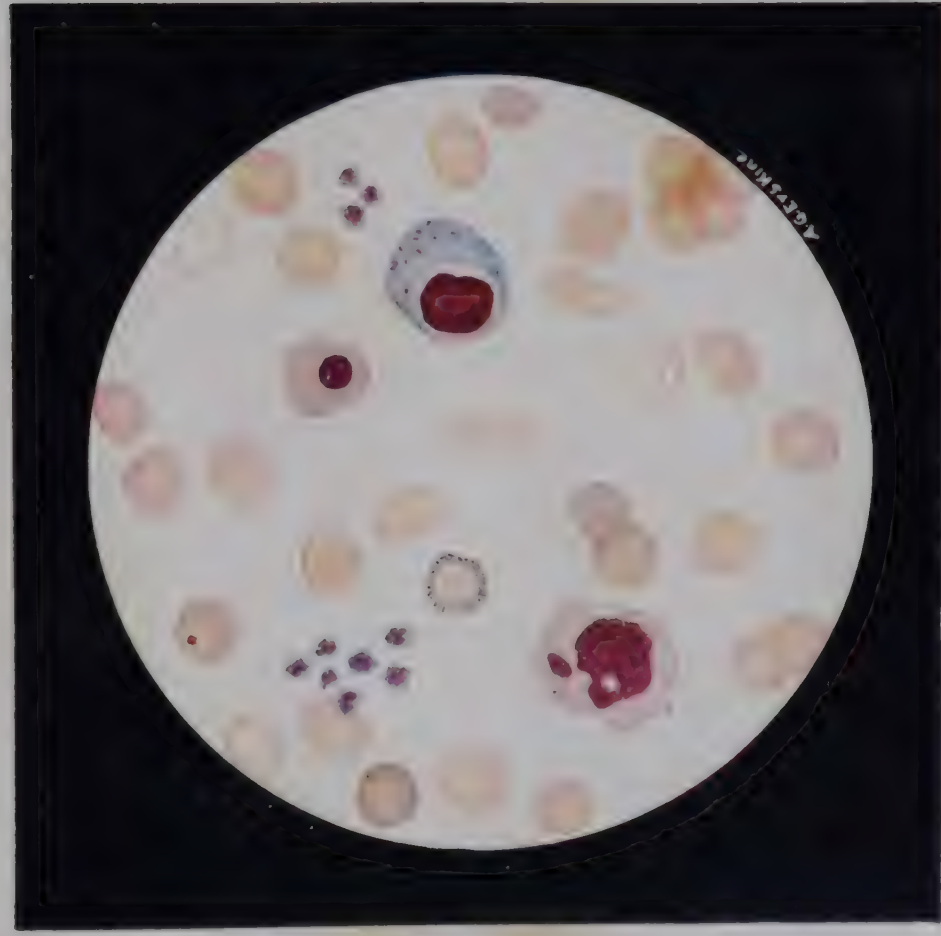


1. Basophilic punctated erythrocytes
2. Eosinophile
3. Lymphocyte
4. Segmented neutrophile
5. Blood platelets
6. Polychromatic erythrocyte
7. Erythrocyte showing marked hypochromia

PLATE XVIII.

HYPOCHROMIC ANEMIA

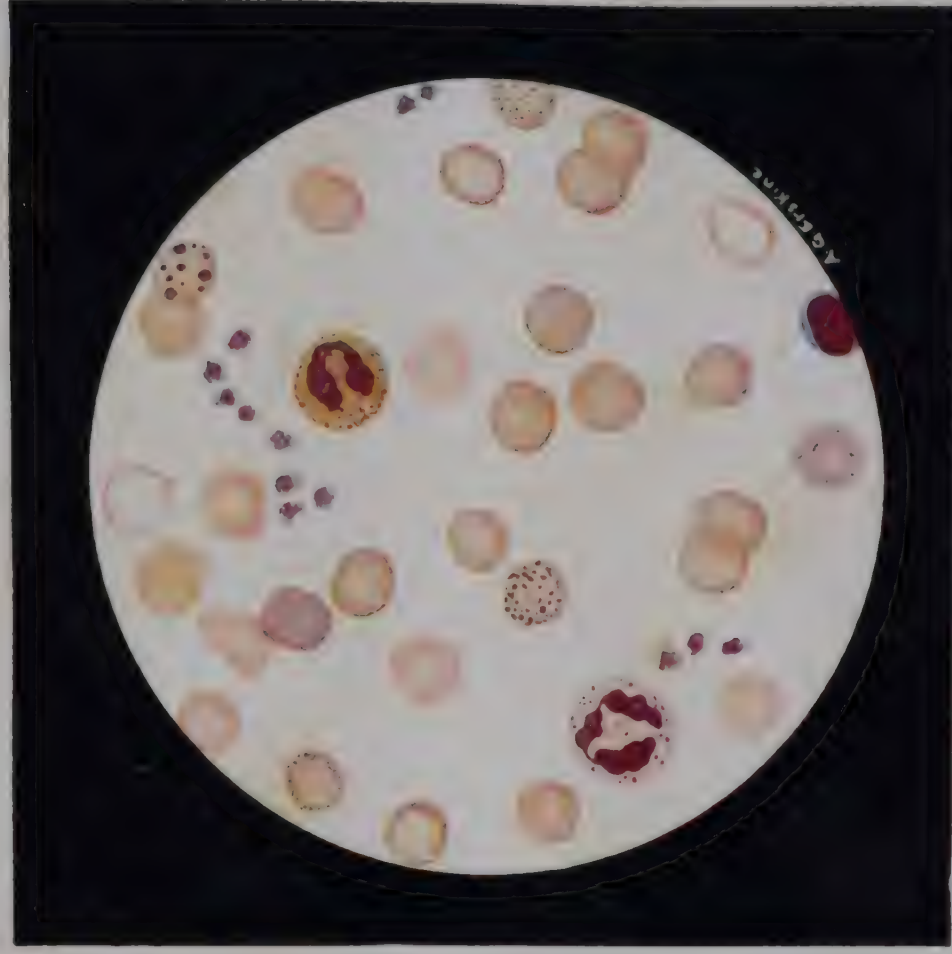
GIEMSA STAIN



X 950

LEAD POISONING

GIEMSA STAIN



X 950

PLATE XVIII.

ance of other symptoms." There is a marked tendency today, with this disease entering into court circles, for litigants to attempt to convince juries, judges, and Workmen's Compensation Boards that the finding of basophilic punctation and polychromasia is proof positive of a case of lead poisoning or vice versa. It is fair to assume in these cases that in addition to basophilia, the entire blood picture must be studied and weighed in the balance, as well as the clinical symptoms, before accurate conclusions may be drawn.

As evidence of the abnormal pigment metabolism related to hemoglobin in lead exposed workers, Bashour and Watson* have demonstrated increased excretion of urinary coproporphyrin and uroporphyrin, as well as the presence of porphobilinogen.

Leukocytic Picture in Lead Poisoning.—Thiele,¹ Seiffert,² and Seitz³ studied the effects of lead on the white blood cells. Thiele claimed that the lymphocytes are destroyed, but this was denied by Seiffert. On the contrary, Seitz called attention to the lymphocytosis in lead poisoning: this has been confirmed by Thiele, Stickl,⁴ Legge-Godbay, Baader⁵ and others. Schmidt-Kehl⁶ noted lymphocytosis in self-experimental work. Stickl⁴ noted also a monocytosis. Nestsadimenko and Volkow⁷ claim that there is a left shift of the neutrophiles. These findings are inconclusive: *we have no justification for describing a characteristic leukocytic picture in lead poisoning.* Since we appreciate fully that the human organism, when afflicted with lead poisoning, must be under the stress of severe nerve and endocrine insult, we may assume that the consequent leukocytic blood picture changes are referable to tissue reactions of this type; an early slight nuclear shift may be due to a toxic irritation by lead; a lymphocytosis and even a monocytosis may be due to a plumbic injury of the myelocytic system. So, too, may we understand the occasional and irregular eosinophilia that has been described by certain workers as characteristic of plumbism. As a matter of fact, Selig⁸ claimed that eosinophilia which he observed in lead poisoning (as high as 70 per cent) was due to a vagus injury.

Erythrocytic Picture of Lead Poisoning.—In addition to the basophilic punctation and polychromasia, it is generally believed that there is a general anemia present in lead poisoning. This was well explained by Aub and Reznikoff. This anemia, however, is not nearly as severe as is generally believed. Teleky⁹ reported hemoglobin percentages from 70 to 80 per cent. Seitz reported a number of cases³ but not more than a fourth of them had hemoglobin less than 80 per cent. That hyperchromemia is a part of lead poisoning has been asserted by some and denied by others. There is no evidence that lead poisoning can produce a hyperchromatic megalocytosis. According to

*Bashour, F. A., and Watson, C. J.: Proc. Cent. Soc. Clin. Res. 27: 10, 1954.

¹Thiele: München, med. Wchnschr., p. 338, 1924.

²Seiffert, G.: München, med. Wchnschr. p. 1580, 1921; p. 1595, 1922.

³Seitz: (a) München, med. Wchnschr. 70: 1501, 1923.

(b) München, med. Wchnschr. 74: 1364, 1927; 75: 1544, 1928.

⁴Stickl: Arch. f. Hyg. 98: 43, 1927.

⁵Baader: Gewerbekrankheiten, Urban & Schwarzenberg, 1931.

⁶Schmidt-Kehl: Arch. f. Hyg. 98: 1927.

⁷Nestsadimenko and Volkow (Hämogramm), Ukrain. Med. Vistl 4: 337, 1924.

⁸Selig: Klin. Wchnschr., Heft 14, 1924.

⁹Teleky: Handbuch der sozialen Hygiene, vol. II, Berlin, 1926.

Naegeli¹ the smallest number of red cells found in a case of plumbism was 3,500,000 by Becker; 3,700,000 by Malassez; 2,200,000 by Limbeck. In his own 300 cases, Naegeli found one case with 50 per cent hemoglobin, 2,832,000 reds, 12,800 whites. Levin² calls attention to one case with hyperchromemia, 65 per cent hemoglobin, 2,800,000 reds, and a color index of 1.16.

The momentous points to be settled in dealing with lead cases are—is there some factor other than lead that can explain the condition present? Second, are all the findings compatible with the effects of lead; are lead effects standardized, pathognomonic and not seen in any other condition? In answering these questions, one must have *more than the blood picture* in order to come to a final conclusion; namely, history, clinical symptoms, and perhaps chemical findings.

It must be conceded in reviewing the effects of lead upon the body that the bone marrow is vitally affected, as proved by the results of experimental research of Aub,³ Speransky and Shlianskaya,⁴ Seiffert and Arnold⁵ and others. Speransky and Shlianskaya noted an increased number of erythroblasts and a disturbed leukopoiesis in guinea pigs. Seiffert and Arnold call attention to the increase in the larger erythroblasts with increasing but abnormal regeneration. Schmidt and Barth⁶ found the largest amount of lead in the body always accumulated in the trabeculae and not in the bone marrow. This was confirmed by Aub³ and his co-worker Fairhall. In Schmidt and Barth's research, there was found a necrobiosis of the nuclei. The lead was found bound by the nuclei. They thought they were on the way to prove that basophilia was of karyogenous origin, but later work by others on bone marrow punctures with the use of combined Wright and Giemsa stains showed perfect nuclear structures with equally perfect basophilic punctation in the cytoplasm. It seems rather dubious to believe that basophilic punctation is a resultant of nuclear disintegration. We stand between the thought that polychromatic and basophilic punctated material is a property of many young cells, and also that young regenerative cells of all kinds may perish in the bone marrow as a result of toxic impressions.

In an attempt to clarify the obscurity of the classification of the plumbic anemia, Ucko and Duesberg⁷ suggest that lead anemia belongs to a special category: "an anemia with disturbed regeneration and formation of porphyrin." They would place lead anemia with pernicious anemia, sharply differentiating it from the methemoglobin-forming and hemolytic anemias. One must classify lead anemia as a toxic central disturbed erythropoietic phenomenon, related to special blood diseases of infection, intoxication, and systemic injury. It must be considered apart from the symptomatic simple anemias because of its degenerative tendencies.

Attention must be called to the fact that the effects of lead on the blood persists long after chemical tests would avail in making the diagnosis, thus

¹Naegeli: München. med. Wchnschr., Heft 5, 1904.

²Levin: Ergebnisse der inneren Medizin und Kinderheilkunde, Bd., 35, 1929.

³Aub, Fairhall, Minot and Reznikoff: Baltimore, 1926.

⁴Speransky and Shlianskaya: Folia haemat. 36: 289-315, 1928.

⁵Seiffert and Arnold: Arch. f. Hyg. 99: 272, 1928.

⁶Schmidt and Barth: Arch. f. Schiffs- u. Tropenhyg. 20: Part 1, 1925.

⁷Ucko and Duesberg: Cited by Schilling: Structure of the Erythrocytes, Folia haemat. 14: 1922. Virchows Arch. 234: 1921; 265: 1927.

adding another pitfall in the path of exact diagnosis. These considerations must be carefully weighed in forensic determinations.

Schilling has done much to demonstrate that technic in preparation of blood specimens is a more important factor than was heretofore believed, in clinching the diagnosis of lead poisoning. Ordinary spreads do not suffice. One must incorporate into routine practice *the making of thick drops in all cases*. There is frequently a marked discrepancy between data from spreads and those from thick drops. In a number of cases here in St. Louis where forensic considerations required examination of the same individual by different workers, we have seen otherwise good and honest testimony weakened by failure to make thick drop examinations in suspected lead cases. Spreads fail to show characteristic basophilic punctation and polychromasia often where thick drops give undeniable evidence of this kind. In 1924 Koch¹ urged that a standard method of staining should be worked out for these cases. For blood films, possibly the Schwarz² modification of the Manson method is the method of choice (page 619). As a matter of fact, the Giemsa and Wright methods of staining ordinary blood films suffice quite well to demonstrate basophilic punctation and polychromasia, as well as all other components of the blood picture.

The **supravital methods of staining** are admirable for the demonstration of basophilic punctation.

The **thick drop method** of Schilling is the best method of demonstrating basophilic punctation and polychromasia in lead poisoning (see page 650). This method, originally introduced by Ross for the demonstration of malarial parasites, is now widely used in lead poisoning studies. An observation of the central part of the drop will show the different degrees of basophilia present.

The average blood picture in lead poisoning would be: P and BP + + +, medium to coarse, torn. Hemolytic icterus: P + + + +, fine to coarse.

There has been some controversy as to just how to interpret this basophilic punctation in blood films and in thick drops. Brueckner claims that no special significance may be attributed to basophilic punctation as compared with polychromasia and that the coarser forms of basophilic punctation are simply younger and perhaps more activated cell forms. Weight of opinion, however, seems to point to the fact that polychromasia and basophilic punctation are quite different. Polychromasia is found in marked degree in many conditions, under an endless variety of pathologic conditions, even in supposedly healthy persons who are probably under the influence of a slowly developing occult chronic illness. Basophilic punctation, on the other hand, is found only in very severe toxic anemias due to blood poisoning in various forms, protozoal disease, purpura, pernicious anemia, leukemia, and aplastic anemia. In other words, it is an entity due to a very definite and serious condition. Where one can reasonably believe that no malaria exists, the first thought in finding a very pronounced state of basophilic punctation in the thick drop or film would be lead poisoning, or poisoning with benzol, arsenic, or with any one of the heavy metals. An objection has been raised to this conclusion by those who have studied the guinea pig experiments

¹Koch: Arch. f. Hyg. 94: 306, 1924.

²Schwarz: Klin. Wchnschr., Heft 44, 1922.

of Lehmann,^{1, 2} but one must remember that guinea pigs and man are quite different, that guinea pigs are quite sensitive and have been known to develop basophilic punctation from poor housing conditions. It must be mentioned here, however, that Konrich³ and Lehmann² report basophilic punctation in cement workers. Jötten and Scharlau⁴ failed to find basophilic punctation in workers in dusty occupations.

The McCord basophilic aggregation test in lead poisoning does not give as much information as the reticulocyte count and is less accurate, and so it has been omitted from this edition.

Summary

Lead poisoning is an acute disease characterized by symptoms involving three systems: namely, the gastroenteric, hematopoietic, and the nervous systems. Rarely the latter may appear to be exempt; the former two never.

Acute episodes may recur and give a false appearance of chronicity. Sequelae also may persist for long periods. Signs of abnormal lead absorption are mistakenly considered to represent illness.

The cure of the disease depends on the cessation of hazardous exposure and the elimination of toxic quantities of lead from the body.

Kehoe⁵ emphasizes this need for prophylaxis and decries the lack of proper preventive measures as indicated by the great number of cases of lead intoxication that still exist. He particularly sounds a note of warning concerning the use or possible "misuse" of the newest agent in the treatment of lead intoxication, namely, calcium disodium versenate (ethylenediamine-tetraacetic acid, EDTA). Kehoe calls attention to the long list of substances which have been used in the past to mitigate the effects of excessive absorp-

TABLE 56.—LEAD LEVELS* IN HEALTH AND IN VARIOUS INDUSTRIES

	NORMAL LEAD LEVELS	SAFE ABNORMAL LEAD LEVELS†	HAZARDOUS ABNORMAL LEAD LEVELS‡
Blood—mg. per 100 gm.	0.01 to 0.06 Average 0.03	0.01 to 0.07	0.07 to 0.4
Urine—mg. per liter	0.01 to 0.08 Average 0.03	0.01 to 0.14	0.1 to 0.8
Stool—mg. per gram ash	0.005 to 0.09 Average 0.03	0.05 to 0.25	0.15 to 2.0
Spinal fluid—mg. per 100 c.c.	0.003 to 0.001 Average 0.001	Less than 0.004	More than 0.004
Stippled red blood cells per 50 oil immersion fields—12,500 cells	0 to 12 Average 4	Less than 16	16 to 500 Average 75
Average daily intake from all sources	0.05 to 2 mg. per day. Average 0.33		

*Acknowledgment is made here to Dr. Robert A. Kehoe, Director of the Kettering Laboratory of Applied Physiology, University of Cincinnati, College of Medicine, for much of the information in this summary.

†Seen in certain industries with abdominal lead exposure at magnitudes not sufficiently great to cause clinical plumbism.

‡Cases of plumbism occur within this range. **NOTE:** Not all cases within this range have clinical plumbism.

¹Lehmann, H.: Arch. f. Hyg. 99: 1928; Zentralbl. f. Gewerbehyg., p. 121, 1931.

²Lehmann, H.: Arch. f. Hyg. 96: 1926; 102: 1921; Zentralbl. f. Gewerbehyg. p. 128, 1931.

³Konrich: Arbeiten aus dem Reichsgesundheitsamt 57: p. 122, 1928.

⁴Jötten and Scharlau: Zentralbl. f. Gewerbehyg. 8: 28, 1931.

⁵Kehoe, R. A.: J. A. M. A. 157: 341, 1955.

TABLE 57.—LEAD ABSORPTION IN CERTAIN TRADES

SUBJECTS	NO. OF SAMPLES	MEAN LEAD CONTENT	
		FECES MG./SAMPLE	URINE MG./LITER
Mexican Indian -----	29	0.11	0.022
Frenchmen (No Industrial Exposure)-----	34	--	0.020
Normal American Adult M.R. -----	31	0.38	0.021
Normal American Adult H.D. -----	548	0.26	0.022
Germans (No Industrial Exposure) -----	13	--	0.027
Normal American Adult E.B. -----	56	0.22	0.031
Medical Students -----	71	0.24	0.038
Garage Mechanics -----	81	0.51	0.047
Garage Mechanics -----	71	0.54	0.050
Garage Mechanics -----	140	0.47	0.051
Unexposed Workmen -----	172	0.47	0.051
Workmen in Paint Mfg. -----	19	0.15	0.051
Workmen in Insecticide Mfg. -----	20	--	0.067
Workmen Engaged in Soldering -----	31	0.51	0.070
Workmen in Tetraethyl Lead Mfg. -----	35	0.47	0.079
Workmen in Tetraethyl Lead Mfg. -----	34	0.63	0.069
Firearms and Bullet Mfg. Workers -----	74	0.48	0.097
Storage Battery Workers -----	21	1.73	0.128
White Lead Workers -----	40	2.70	0.176
White Lead Workers -----	26	4.00	0.191
Brass Foundrymen -----	46	2.78	0.250
Storage Battery Workers -----	35	0.62	0.201
Storage Battery Workers -----	9	3.72	0.330
White Lead Workers -----	10	7.60	0.336

tion of lead, such as milk, ascorbic acid, laxatives and purgatives with lemonade fortified by acid. Although the new derivatives of edathamil appear to have the property of combining with lead in the soft tissues and then being excreted in the urine, it has not been established that it is lifesaving or that it can be relied upon to relieve symptoms. For the relief of colic Vigliani² recommends that cortisone or corticotropin be administered along with drugs which either neutralize or eliminate lead from the body. Belknap and Perry³ recommended the combination of intravenous calcium gluconate and edathamil calcium disodium for the treatment of lead colic.

Lead Intake and Output of Normal Subject (M.R.) During 4-Year Period of Oral Administration.

	MG.	% OF TOTAL INGESTED
Ingested:		
In drinking water -----	30.00	1.5
In food and beverages -----	498.47	25.3
Administered in solution -----	1442.80	72.2
	-----	-----
Total -----	1971.27	100.00
Excreted:		
In feces -----	1739.31	88.2
In urine -----	113.77	5.8
	-----	-----
Total -----	1853.08	94.0
	-----	-----
Retained: -----	118.19	6.0

²Vigliani, E. C.: Arch. Indust. Hyg. 10: 491, 1954.
³Belknap, E. L., and Perry, A. M.: Arch. Indust. Hyg. 10: 530, 1954.

Symptoms of Lead Poisoning:*

100% generalized weakness
90% weight loss
82% constipation
80% colic
77% anorexia
77% abdominal pain
70% arthralgia
57% lassitude
54% generalized aching
54% vomiting
50% nausea
43% insomnia
40% headache
40% metallic taste
30% abnormal dreams
27% excessive salivation
27% vertigo
10% impotence

Physical Signs in Lead Poisoning:*

66% lead line
66% pyorrhea
60% dental decay
57% wrist extensor weakness
40% malnutrition
37% abdominal tenderness
37% infected tonsils
33% hyperactive biceps reflex
33% hyperactive abdominal reflexes
33% hyperactive cremasteric reflexes
30% tremor
27% hyperactive tendon reflexes of legs
27% pallor
20% sensory changes

Vital Signs and Clinical Laboratory Data:

Temperature, 98.4° F. Pulse, 80. Respiration, 22. Blood Pressure, 132/79. Red Blood Count, 4,270,000. White Blood Count, 7,550. Hemoglobin, 11.4 gm. Urine—occasional cast.

Effects of Other Chemicals on the Normal Blood Picture

In addition to lead, the blood picture may be altered by other chemical substances. Acetanilid, acetphenetidin, and other aniline derivatives produce anemia. Stengel and White¹ reported a case of chronic acetanilid poisoning and severe anemia with red cells 2,000,000 and hemoglobin 35 per cent. There were present a great many normoblasts with many variations in the size and shape of the red cells.

Arsenic in small amounts over a long period of time causes a mild stimulation of hematopoiesis. When arsenic is ingested in toxic amounts, there is a reduction in the granular leukocytes and actual leukopenia may occur.

Arsphenamine in exceptional cases has produced an effect upon the bone marrow very similar to that seen in aplastic anemia or purpura hemorrhagica. This effect may be fatal.

Carbon monoxide produces secondary anemia. In acute poisoning, the hemoglobin changes to methemoglobin.

Nitrobenzene, or shoe dye poisoning, has been seen frequently. It is not at all certain that the union of the poison with hemoglobin produces methemoglobin, so that the term "pseudomethemoglobinemia" has been devised by Peters and Van Slyke² for this condition. There are no changes in the morphology of the red cells.

*From a statistical analysis of 30 cases.

¹Stengel, A., and White, C. Y.: Univ. of Penn. Med. Bull. 15: 462, 1903.

²Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry, Vol. I, Interpretations, Baltimore, The Williams and Wilkins Co., 1931.

Poisoning by Benzol and Related Products

Benzol is a volatile inflammable fluid, used in industry for cleaning printing rooms and engraving plates, in rubber cements, in lacquers, varnishes, and for many other purposes, it being an excellent solvent for rubber, gums, fats, and resins.

Acute poisoning is often due to breathing excessively large amounts of benzol vapors. This may be rapidly fatal. Acute poisoning more often occurs as a result of an accident, such as the bursting of a pipe line, the overflow of a large tank, or workmen entering a tank for cleaning or repair work without using proper protective measures.

Chronic poisoning differs from the acute type in that benzol is inhaled in small quantities over a considerable period of time, the chief effects being observed in blood and blood-forming organs. It is a slow process and the early stages and symptoms are not striking. Later, the symptoms increase, and if the case is not recognized and exposure continues, serious poisoning may develop, which, in advanced cases, may produce death.

Acute poisonings, for instance, deaths in garages, poisoning in tanks, due to benzol (Marienfeld² and Zangger³) show no specific hematologic change. The subacute and the chronic benzol poisoning are suggestive of idiopathic blood diseases, and it is very difficult to distinguish them from thrombopenic diseases, such as Werlhof's purpura, agranulocytosis, aplastic anemia, aleukia, panmyelophthisis, etc.

The first communication on this subject was made by Santesson,⁴ who reported four fatal cases of women workers in a velocipede factory; all four cases were among young women workers.

CASE 1.—Eighteen-year-old girl worked only three weeks in the factory, became sick with fever, pulse 120, and had severe anemia with hemorrhagic tendency. Death followed after several weeks while she was sitting at her work. At autopsy nearly all the organs showed small or large hemorrhages.

CASE 2.—A woman, aged 20 years, who worked four months in the factory, showed first signs of hemorrhagic tendency before giving up her work. She came down with severe bleeding from teeth and nose and vomited blood. Death followed with a severe anemia one week after she went to bed.

CASE 3.—Nineteen-year-old woman worker, who worked two months in the factory, showed hemorrhagic diathesis for three weeks. Her hemoglobin dropped to 20% and red cells to 600,000. Four to five weeks after quitting work, the patient died with outspoken hemorrhagic tendencies.

CASE 4.—Nineteen-year-old woman worker had worked four weeks in the factory. There was a severe leukopenia with hemoglobin of 80%, red cells 3,764,000. Death occurred, with symptoms of hemorrhagic diathesis, one and one-half months after quitting work.

All these cases began clinically with the hemorrhagic diathesis of the skin, blue spots, hemorrhages from the gums and nose, and sometimes intestinal bleeding. In other words, a picture of purpura was seen in all cases.

²Marienfeld: *Ärzt. Sachverständigen-Ztg.*, Bd. 34, 1928.

³Zangger: *Deutsche med. Wchnschr.*, 1929.

⁴Santesson: *Arch. f. Hyg.* Vol. 31, 1897.

Santesson noted that with pure benzine experimentally, no results were obtained, while with benzol (benzene), the same result was seen as in these four cases. In the autopsy material a study showed that severe endothelial fatty changes were responsible for the bleeding. Santesson believed that there was a central disturbance, which was first noted by Selling¹ whose clinical experimental work has been noted so often in the literature. Santesson reported two girls with chronic benzol poisoning, the first showing 640,000 erythrocytes and 8% hemoglobin. There was a severe thrombocytopenia. In the second case there were 1,150,000 erythrocytes with 15% hemoglobin and no regenerative forms.

Selling states that thorium X and benzol act upon the bone marrow as poison, leading to severe cellular diminution in the bone marrow substance, that is, deprivation of the specific parenchymatous cells. The erythroblasts and myeloblasts are disturbed, while the peripheral red cells are also destroyed. There is a slight aplasia of the lymphatic organ. The lymphocytes in the peripheral circulation and the polyblasts in the tissues seem to be most resistant to the actual poison. We find very few myelocytes and normoblasts in the circulation. We find usually severe leukopenia. After daily injections of from 0.5 to 3 c.c. of benzol in rabbits, in six to ten days the leukocytes practically disappeared. The blood picture became composed almost entirely of lymphocytes and monocytes which were absolutely decreased, relatively naturally increased. Selling,² as well as Pappenheim,³ spoke of the peripheral solution of blood. Veit⁴ established a degenerative disturbance of the lymph centers while Pappenheim had noticed an irritation in the parenchyma of the lymph tissue. Neumann⁵ described marked differences experimentally among various poisons. He found in the bone marrow a failure of myelocytes and giant cells but saw areas of pigment composed of destroyed erythrophages, and a definite aplasia of the malpighian corpuscles in the spleen. Later these researches were taken up with better technic by Silberberg,⁶ Woronow,⁷ and Orzechowski.⁸ Silberberg found in rabbits an absolute leukopenia after giving 3 c.c. of benzol subcutaneously.

A few monocytes are found in the blood picture. Woronow noted an aleukia on the seventh to eleventh days, otherwise about 300 to 500 leukocytes in severe cases. Selling spoke of hyperleukocytoses which other workers after him did not establish. Shortly before death in experimental rabbits Woronow noted a liberation of from 10,000 to 15,000 leukocytes, then, right before death, a neutropenia.

These differences in the results are due either to differences in the individualities of the patients, or animals, or because there were some poisons with impure benzol, that is, xylol or toluol, etc. Woronow established that with xylol and toluol, after beginning neutropenia, hyperleukocytosis dropped;

¹Selling: Beitr. z. pathol. u. z. allg. Path. 51: 576, 1911.

²Selling: Beitr. z. pathol. u. z. allg. Path. 51: 576, 1911.

³Pappenheim: Ztschr. f. exper. Pathol. u. Therap. 15: 1914.

⁴Veit: Beitr. z. pathol. Anat. u. z. allg. Path. 68: 425, 1921.

⁵Neumann: Deutsche med. Wchnschr. p. 394, 1915.

⁶Silberberg: Virchows Arch. f. path. Anat. 267: 483, 1928.

⁷Woronow: Virchows Arch. f. path. Anat. Bd. 271: 1929.

⁸Orzechowski: Virchows Arch. f. path. Anat. 271: 191, 1929.

with xylol, monocytes 40% (absolute count 13,000); with toluol, monocytes 30% (absolute count 5,000). Von Oettingen, Neal, and Donahue,⁴ in their work on the toxicity and potential dangers of toluene, showed no such changes in the blood picture. Greenburg, Mayers, Heimann, and Moskowitz,⁵ in a study of 106 painters exposed to the inhalation of toluene of between 100 and 1,100 parts per million for periods of time ranging from two weeks to more than five years, found that such exposure produced moderately decreased levels of erythrocytes, increase in absolute lymphocyte counts, but such exposure did not result in leukopenia. They found the best evidence of early chronic toluene intoxication in man to be enlargement of the liver, and macrocytosis. Woronow noted a very marked shift over to myelocytes and found basically stained granules in the neutrophils and their predecessors.

While Duke⁶ considered the blood platelets as the most sensitive element in these instances of isolated thrombocytopenia, Orzechowski believed that the blood platelets remained normal. As a rule, according to Brücken,⁷ and Bock and Wiede,⁸ the blood platelets are severely affected. All the investigators note an extraordinary hyperemia of the bone marrow, which becomes deep red, as a result of overfilling of the venous sinuses and the presence of very few cellular elements except a few mesenchyme cells, monocytes, lymphocytes, and plasma cells.

Differential Characteristics of the Anemias

Table 58 was developed at the Gradwohl School of Laboratory Technique to aid in teaching the laboratory findings in some of the anemias. It is not intended to be a complete review of the various anemic conditions, but simply a handy reference.

Pseudoanemia (Sahli)

Pseudoanemia of Sahli has been called by the Germans "Schein" or "apparent" anemia. It is a form of anemia well described by Baar and Stransky.⁹ Affected children appear pale but there is no diminution in the amount of hemoglobin in their blood. The blood has a normal composition. The face is pale, the visible mucosae are pale and the ear lobes when viewed through transmitted light are pale. This condition is frequently seen in school children. These children are known as "poor eaters" complaining of weakness, malaise, cold hands and feet. Mueller¹⁰ has divided these cases into three groups:

1. Neuropathic children with disturbances in their vagi and sympathetic nervous system. This leads to a contraction of the peripheral small cutaneous vessels, with consequent pallor of face.

⁴von Oettingen, W. F., Neal, P. A., and Donahue, D. D.: J. A. M. A. 118: 579, 1942.

⁵Greenburg, L., Mayers, M. R., Heimann, H., and Moskowitz, S.: J. A. M. A. 118: 573, 1942.

⁶Duke: J. A. M. A. 65: 1915.

⁷Brücken: Deutsche med. Wchnschr. p. 1120, 1923.

⁸Bock and Wiede: Fol. haemat., Bd. 42, 1930.

⁹Baar and Stransky: Die klin. Hemat. d. Kind, p. 174.

¹⁰Mueller, E.: Jahrb. f. Kinderh. 72: 176-208.

TABLE 58.—CHART OF THE DIFFERENCES

	BEGINNING PERNICIOUS ANEMIA	ADVANCED PERNICIOUS ANEMIA	PERNICIOUS ANEMIA UNDER TREATMENT	CHLOROSIS (IRON DEFICIENCY ANEMIA)	APLASTIC ANEMIA
Erythrocyte count	Slightly low- ered	May be as low as 1,500,000 or lower	Let it go above normal as a rule in treat- ment	Around 4,000, 000 (except in very severe cases)	Progressively erasing red cou
Hemoglobin	Slightly low- ered	Drops quite low but not as much proportionately as drop in red count	Rises under treatment	Very low 50- 60%	Progressively ered. About s rate as red co.
M.C.V.* cu. μ	Slightly over 94	100 or higher	80 to 94	Less than 80	80 to 94
Blood vol- ume	Low	Below normal	Below normal	Very low	Very low
M.C.H.C.† %	Slightly above 30	Slightly above 30	Greater than 30	Less than 30	Less than 30
Leukocyte count	Slightly lower, if any change	Leukopenia (3000-4000)	Rises back to normal	Usually higher, around 10,000	Progressively lowered
Differential	Very little sig- nificant change. Any change will be shift to the right	Leukopenia, neu- tropenia, right shift, hyperseg- mented neutro- philes, lymphocy- tosis, monocytosis. In very serious cases may see myelocytes	Gradual return to normal	Lymphopenia; frequently a "stab" shift	Normal at beg- ning without young cells. 1 small lympho- cytes up to 10 Never any im- ature white cell
Reticulo- cytes	Increase. Go higher after liver treat- ment	Depends upon the amount of regen- eration in patient	Begin to rise on 4-5th day and continues to rise until 10th day. Drops to normal	Increased	Usually have no at all
Blood plate- lets	Begin to de- crease	Low absolute	Begin to rise	Normal	May completely disappear
Red cells in spread	Polychromasia. Tendency of erythrocytes to enlarge	Anisocytosis with macrocytes. Poikilocytosis. Polychromasia. Basophilic punc- tation, hyper- chromia, macro- cytes and megalo- cytes, nucleated red cells; may find Howell-Jolly bodies, Cabot rings, marginal granules, erythro- cytes, etc.	Return to nor- mal. Increase in polychro- masia. Disap- pearance of pathologic erythrocytic findings, as a good sign	Very marked hypochromia. Poikilocytosis, anisocytosis, many micro- cytes, diffuse basophilia; normoblasts and micro- blasts may at times be present	Normocytic Normochromic No change in rocytes noted
Size of red cells	Majority are normal. Tendency to enlarge	Large	Return to nor- mal	Small	Normal
Pigment content of red cells	Not signifi- cantly changed	Hyperchromic (Increased)	Return to nor- mal	Decreased	Normal

*Mean corpuscular volume.

†Mean corpuscular hemoglobin concentration.

CHARACTERISTICS OF THE ANEMIAS

MEMOR- ANEMIA ON-RED	SICKLE CELL ANEMIA	CHRONIC HEMOLYTIC ANEMIA (IDOPATHIC)	LEAD POISONING	VON JAKSCH'S DISEASE
is on se- of hem- ge. Rises ood sign	Around 3,000,000	2-3,000,000 during attacks	2½-3,000,000	1½- 2,000,000
is on se- Usual- le low	Quite low, about same rate as red count	Drops proportionately to red cells	Very low. Can go as low as 20%	Very low. Ratio of drop not constant
han 80	80 to 94	80 to 94	Variable	Over 94
	Low	Low	Low	Low
han 30	Less than 30	Less than 30	Variable	Over 30
10-15,000	High	High 25-30,000	Indefinite	Extremely high
ophilia out a shift	shift. Frequently re- generative	Very marked regenerative shift	Indefinite	Myelocytic leukemic blood pic- ture; at times lym- phocytic
rapid in- e—de- ng on d manu- ure	Quite an increase	Very high	Extremely high	Increased
ase as a sign	Very high	Very high	No significant change	Fairly low
see aniso- sis. ked poly- masia as a sign. eated reds severe orrhage aloblasts serious	Anisocytosis, poikilo- cytosis, polychromasia, normocytic normo- chromic. Nucleated red cells. Basophilic punc- tation. Howell-Jolly bodies. Cabot ring bodies, endoglobular de- generation. Target cells. Sickling does not appear in film as a rule	Anisocytosis, poikilocytosis, marked polychromasia, many normoblasts, baso- philic punctation. Cabot ring bodies, Howell-Jolly bodies, marginal granules, karyorrhexis, karyogenic metachromasia, megalo- blasts, hypochromia, endo- globular degeneration, crescents	Marked poly- chromasia. Basophilic punctation, very coarse. May find other pathologic erythrocytes, such as normo- blasts, etc.	Very much like ad- vanced pernicious anemia
cal, large, ay be ll	Normal	Normal	Indefinite	Large
ly low	Normal	Normal	Indefinite	Hyperchro- mic (in- creased)

TABLE 58.—CHART OF THE DIFFERENTIAL

	BEGINNING PERNICIOUS ANEMIA	ADVANCED PERNICIOUS ANEMIA	PERNICIOUS ANEMIA UNDER TREATMENT	CHLOROSIS (IRON DEFICIENCY ANEMIA)	APLASTIC ANEMIA
Special ex- aminations needed	Blood volume and volume index. Reticu- locyte count, blood platelet count, van den Bergh, bone marrow punc- ture, gastric analysis	Reticulocyte count. Routine complete blood examina- tion. Same as beginning per- nicious anemia	Reticulocyte counts repeat- edly. Repeated red cell counts and hemo- globin	Complete blood picture	Bone marrow punc- ture. Routine hematology serially
Bone mar- row	Increase in megaloblasts	Great increase in megaloblasts. Decrease in granulocytes	Return to nor- mal, first show- ing increase in normoblasts	Not needed for diagnosis	Either absence of nucleated reds or great decrease. Only an occasion- al normoblast. May be fatty de- generation or in- filtration of sma- ll lymphocytes. Granulocytes dis- appear. May be plasma cells
Classifica- tion	Normocytic normochromic with macro- cytes	Macrocytic normo- chromic	Return to nor- mocytic nor- mochromic	Microcytic hy- pochromic	Normocytic normochromic
Important points in diagnosis	Increase in size of red cells. Increase in reticulocytes. Response of patient to treatment with erythro- cyte matura- tion factor (liver, vita- min B ₁₂)	Very low red count and hemoglobin and high color index. Low white count, right shift and hypersegmen- tation of neutro- phils. Megalo- cytes and megalo- blasts, hyper- chromia; increase in reticulocytes following liver therapy; low blood platelets	Reticulocyte re- sponse to liver treatment. Re- turn of blood picture to nor- mal	Tiny red cells with decreased pigment con- tent. Low col- or index	Entire blood and bone marrow pic- tures taken seria- ly, giving pictur- e of true aplasia

2. Abnormal distribution or disproportion between blood supply in various parts of the body; that is, a pallor not due to cutaneous vessel contractions but rather to diseases of the internal organs whereby a greater volume than normal remains in these organs.

3. "Local" cases; that is, pallor is due to apparent change in the transparency of the skin, a poorly developed capillary vessel network in the skin

Aron³ explains the first group on the basis of a poorly developed circulatory system; that is, a relative insufficiency of the heart. He claims that these cases show an abnormally low Livi Index (Index ponderabilis) which obtained by mathematical computation—

$$\sqrt[3]{\frac{\text{Body weight} \times 100}{\text{Body length}}}$$

³Aron: Jahrb. f. Kinderh. 87: 873, 1918.

CHARACTERISTICS OF THE ANEMIAS—CONT'D

THROMBOCYTIC ANEMIA	SICKLE CELL ANEMIA	CHRONIC HEMOLYTIC ANEMIA (IDIOPATHIC)	LEAD POISONING	VON JAKSCH'S DISEASE
Reticulocyte count, serial count, blood platelet count, hemoglobin test	Wet preparation. Sometimes mix 1 drop patient's serum plus one drop patient's cells. Sodium metabisulphite test and alkali denaturation test, electrophoretic studies	Reticulocyte count, resistance test, van den Bergh, icterus index, blood platelet. Bone marrow puncture	Chemical analysis of urine and feces. Reticulocyte count; thick drop	Age of patient. X-ray
Decrease in normoblasts and monophiles	Regenerative—packed with young red and white cells	Hyperplastic erythropoietic	In some cases, basophilic stippling. Not needed for diagnosis as a rule	Not needed
Normocytic hypochromic, normocytic normochromic	Normocytic normochromic	Normocytic normochromic	Indefinite	Often macrocytic normochromic
Increasing red count and hemoglobin, increase in reticulocytes, high blood platelet count, good signs	75-100% of red cells are sickle-shaped in wet preparation. Electrophoresis shows hemoglobin S. Fetal hemoglobin increased	Entire blood picture and special tests needed. Search for iso- and auto-immune bodies	History of exposure to lead, clinical symptoms, chemical analysis of urine and feces, +++ and ++++ polychromasia in thick drops. Basophilic punctation	Age of patient, combination of leukemic and anemic blood pictures

THE ERYTHROCYTOSES

TABLE 59.—THE ERYTHROCYTOSES*

TERM TO BE USED	TERMS TO BE AVOIDED
Erythrocytosis, due to Polycythemia vera	Erythremia, polycythemia Vaquez's or Osler's disease, erythremia, splenomegalic polycythemia, Gaisböck's disease, polycythemia hypertonica, erythroleukemia, polycythemia rubra vera, erythrocytosis megalosplenica Erythrocytosis, erythremia, erythroleukosis
Arterial anoxemia, due to Unknown cause Heart disease Pulmonary disease Arteriolosclerosis in the lungs Hereditary leptocytosis (trait)	Ayerza's disease

*Reproduced from the Am. J. Clin. Path. 20: 562-571, 1950, by courtesy of the Editor and of the Williams and Wilkins Company, Baltimore.

Polycythemia (Erythrocytoses)

Polycythemia vera was described by Vaquez¹ in 1892 and Osler² in 1903. It is also called "erythremia." There is an enormous increase in the number of erythrocytes, abnormal distention of the blood vessels—or plethora—an enlargement of the spleen. There are hyperactivity of the myeloid tissue and the appearance of many young forms of erythrocytes. There is, as a result of this abnormal disproportion of cells and plasma, instead of 56 per cent of plasma, only about 10 to 20 per cent. These patients show a characteristic reddish complexion, due to the distention of the peripheral vessels of the face. The visible mucosae and the throat mucosa appear a dense purplish red instead of the pale red of an inflammatory condition. The face, hands, and feet feel abnormally warm. By reason of the distention, the capillaries in the face become widened and through local stasis, a cyanosis is apt to occur. In addition to these symptoms, there is an albuminuria and nephropathy. A few casts are found. The blood pressure is elevated. There is likely to be dilatation of the heart, weakness of the heart muscles and hydrops.

Subjective symptoms are headache, dizziness, vomiting, dyspnea, and sleepiness. Occasionally abdominal pains, especially in the left hypochondrium, are noted.

Hirschfeld and Stern call attention to the lowered temperature. Psychic and nervous symptoms are seen.

Polycythemia vera is generally considered a primary disease of the hematopoietic system of unknown etiology. There is evidence of increased activity of the bone marrow, as can be deduced from presence of immature cells in the peripheral blood and the occurrence of polychromasia, normoblasts, and myelocytes in the peripheral blood.

Studies have shown that the stomachs of man, dogs, swine, and cattle contain a substance which is a powerful stimulant of marrow activity in pernicious anemia. It is dialyzable through collodion and withstands esterification, properties that would seem to remove it from the enzymes. It is probably a hormone, for which the name "addisin" has been proposed. Pernicious anemia seems to be due to a lack of secretion of addisin. Theoretically, erythremia may be due to hypersecretion of addisin or hypersusceptibility of the bone marrow to it. This theory was advanced by Morris.³ In a patient with erythremia, having 8.9 million red cells, addisin derived from 1,250 c.c. of the gastric contents of swine was injected intramuscularly. On the fifth day following the injection, the reticulocytes, 1 per cent in amount, rose to 2.5 per cent. On the ninth day after the injection, a differential count showed 1 per cent myelocytes. This result indicated a response of the bone marrow to stimulation by addisin.

Examination of the sources of hematopoietic substances effective in pernicious anemia shows a high purine content (liver, kidney, stomach, nucleic acid).

¹Vaquez, L. H.: *Bull. med. Par.* 6: 849, 1892.

²Osler, Wm.: *Am. J. M. Sc.* 126: 187, 1903.

³Morris, Roger S.: *J. A. M. A.* 101: 200, 1933.

If pernicious anemia represents anaddisinism and erythremia represents hyperaddisinism, it would be theoretically possible to control erythremia by dietary measures. For this reason, Morris proposed a low purine diet continued over a long period of time for the treatment of erythremia.

Relative polycythemia* occurs when, through loss of blood plasma, the concentration of the red corpuscles becomes greater than normal in the circulating blood. **Transient polycythemia** occurs when in response to some stimulus red corpuscles are shunted into the circulation from some storehouse such as the spleen. **Absolute polycythemia** means an increase which is associated with an increase in the total red cell mass.

Absolute polycythemia may be divided into two forms, erythremia and erythrocytosis. Erythrocytosis thus denotes a polycythemia which occurs in response to some known stimulus, whereas erythremia refers to a disease of unknown etiology.

Relative polycythemia may be caused by dehydration and may give the patient the appearance of an absolute polycythemia. In this condition, however, there is a diminished blood volume but no real overproduction of red blood cells. This occurs following excessive exercise in which there has been an unusual excretion of water through the skin and lungs. Prolonged vomiting, diarrhea, and fever may be causative factors. In other words, it is due to loss of water from the blood plasma following abnormally lowered fluid intake or marked loss of body fluids. The differential diagnosis depends upon the presence of increased red blood cells associated with diminished blood plasma volume as determined by the hematocrit. The condition is usually transitory, definite improvement occurring by dilution of the blood with fluids.

Transient polycythemia occurs when contractions of the spleen throw red blood corpuscles into the circulation in response to such stimuli as exercise, anoxemia (low oxygen tension, hemorrhage, suffocation, carbon monoxide poisoning), certain drugs, emotional conditions which produce an increase in the excretion of adrenaline into the circulating blood.

Erythrocytosis is analogous to erythremia except for the fact that it is secondary to a known cause and, as generally conceded, usually represents an effort on the part of the organism to compensate for difficulties in the oxygenation of the blood or tissues of the body, the underlying cause of the increased blood production being oxygen want. First, there may be a defective saturation of arterial blood with oxygen as evidenced by the erythrocytosis of high altitude (mountain sickness). This occurs not only in people who live in high altitudes but in travelers as well who go up in planes or climb mountains. Blood counts may reach 7.5 or 8 million cells per cu.mm. This condition from a similar cause (that is, poor oxygenation) is also found in the newborn, congenital heart disease, certain forms of acquired heart disease and pulmonary diseases, and in those conditions due to a defect in the hemoglobin of the blood so that it is unable to combine with oxygen in normal amount as manifested by the toxic action of various bacterial and chemical poisons on the red blood cells.

*This section to the bottom of page 774 is an excerpt of an article by Meyer, H. M.: Lab. Digest 5: 4, 1941.

Under these conditions red cell counts as high as 13.9 million have been recorded, hemoglobin as high as 23.7 gm. (normal, 13.0 to 17.3 gm.), while the volume of packed red cells averages 54.4 c.c. per 100 c.c. (normal, 41.9 c.c. to 42.9 c.c.).

Erythremia or **polycythemia vera** is a slowly and intermittently progressive disease of unknown etiology characterized by a striking absolute increase in the number of red blood corpuscles, and in the total blood volume, and frequently by signs of generally increased bone marrow activity.

Attention was called to this disease in 1892 by Vaquez, some time following the development of a method for counting red blood corpuscles in the middle of the nineteenth century.

The disease is more common in males than females; a fair percentage of the cases are found in Jews born in Eastern Europe. It usually occurs in middle or late life. Several persons of the same family may be affected. The majority of workers in this field believe that the pathologic physiology is an increased activity of the blood building apparatus.

Patients are usually vague in their statements concerning the date of onset of their symptoms. From histories of slight discomfort or vague ill-defined complaints it is evident that the disease is likely to have been present for a long time before it finally obtruded itself upon the consciousness of the patient. Individuals may apply to their physician for cosmetic reasons, to be rid of the unnatural red flush of the skin, and may be otherwise symptom free. Such general symptoms as weakness, lassitude, easy fatiguability, loss of weight, and shortness of breath are frequently present. Various gastrointestinal symptoms including abdominal pain, eructations of gas, anorexia, nausea and vomiting may occur. Skin and mucous membrane hemorrhages are not uncommon. Nervous symptoms manifested by headache, dizziness, noises in the ears, tingling sensations of hands and feet, staggering gait, muscular weakness, blurring of vision, thickness of speech, and even loss of consciousness may be complained of.

On physical examination one finds the characteristic reddish-blue color of the skin and mucous membrane, or there may be a definite pallor. The spleen is enlarged in the majority of cases; the size of the spleen varies greatly at different periods of the disease. Enlargement of the liver occurs in more than 50 per cent of the cases. Blood pressure is usually within normal limits. Muscular twitchings or paralysis may be present. There is no enlargement of the heart in the majority of the cases. Palpitation is complained of by many patients. The disease may be latent for years. After it has become fully manifest, its course is chronic, slowly progressive, and ultimately fatal. Patients may live over a period of from four to twenty years or more after the onset of the recognized symptoms. Death usually results from vascular complications, especially cerebral thrombosis or hemorrhages. Serious and often fatal hemorrhages have occurred from esophageal varices, the stomach, lungs, and other viscera. Heart failure may be the terminal event.

Examination of the blood reveals a marked increase in the red cell count. It usually ranges from 7 to 12 million per cu.mm., the highest authentic count recorded being 13,600,000 per cu.mm. It has been calculated theoretically that with red cells of normal size the circulatory system would be unable to con-

tain the blood if the count exceeded 13,900,000 per cu.mm. The blood is characteristically dark and spreads slowly under the cover glass. It may be so thick that it is drawn up in a pipette with difficulty. Patients with 80.0 c.c. of packed red cells per 100 c.c. of blood have been observed.

In polycythemia the red corpuscles are often normal in size, but it is not uncommon to find that their mean volume is less than normal. Thus the average size in 14 cases was 83 cu. microns—the smallest cells being about 61 cu. microns.

The hemoglobin is also increased, but less than the red cells, so that the color index is ordinarily below 1, the usual range being 0.7 to 0.9. Hemoglobin values as high as 240% have been recorded; this would be equivalent to possibly 40 gm. of hemoglobin per 100 c.c. of blood, but it is more than likely that the hemoglobin determination in this case was not accurate. Hemoglobin values varying from 120 to 160%, or 18 to 24 gm., are common.

The blood counts vary appreciably from day to day; spontaneous remissions and relapses occur. Simultaneous blood counts from venous and arterial blood show in general no marked differences.

The red corpuscles as examined in the blood spread may appear quite normal; however, it is not unusual to find somewhat greater variation in size than is normal, and more rarely, poikilocytosis. Microcytes are not infrequent; macrocytes are unusual. Especially when the disease has become more advanced and particularly when hemorrhage has occurred, the red corpuscles appear paler than normal. Polychromatophilia, and less frequently basophilic stippling, may be encountered. Nucleated red corpuscles are not rare, but other indices of increased erythropoiesis are almost never encountered. Anisocytosis, poikilocytosis, polychromatophilia, and normoblastosis may be quite marked. Special staining reveals frequently a slightly increased reticulocyte count, often about 1 to 2 per cent. Great increases in reticulocytes are found only following hemorrhages.

Leukocytosis is quite common, the count varying from 10,000 to 25,000, but values up to 50,000 per cu.mm. are by no means rare. The leukocytosis is due to an absolute increase in the number of granulocytes. The cells of the myeloid series compose 70 to 95% of the leukocytes. Metamyelocytes are found in increased number, and it is not unusual to find 1 to 2% myelocytes; myeloblasts have also been observed. The eosinophilic and basophilic leukocytes may be increased, although this is less common. Lymphocytes are relatively reduced but usually their absolute number is about normal. Monocytes may be absolutely increased.

The blood platelets are usually increased; counts of 1,000,000 per cu.mm. are quite usual, and in some cases the count may exceed 3,000,000. Megakaryocytes are rarely found in the circulating blood.

Bleeding time is usually essentially normal, and coagulation time is normal. Clot retraction may be slow. The fragility of the red cells may be normal but is frequently increased. In dilutions of their own or normal serum, patient's cells might yield an abnormally long resistance span. Normal cells, however, may be hemolyzed in dilutions of patient's serum.

The viscosity of the blood in polycythemia is much greater than that of normal blood. It may be five to eight times greater. This is primarily due to

the great excess of red blood cells over plasma. The viscosity of the serum, indeed, is said to be less than is normally found in health. Likewise the specific gravity of erythremic blood is 1.075 to 1.080 as compared with 1.055 to 1.065 for normal blood. The sedimentation rate of polycythemic blood is very greatly delayed.

The total volume of blood in erythremia as measured by both the dye and carbon monoxide methods is found to be greatly augmented. Volumes of 121 to 246 c.c. per kg. of body weight have been found as compared to a normal value of 87 c.c. Plasma volumes are increased little or not at all. There is an increase in the blood in the organs in general, and even the smaller vessels of the pia mater of the brain and cord are engorged and distended with blood.

Sternal bone marrow puncture reveals a dark red marrow crowded with enormous numbers of mature erythrocytes as well as normoblasts. All the nucleated red corpuscles are small and contain round, ripe, and often pyknotic nuclei. Megaloblasts are frequently few or absent. Polychromatophilic red cells are few. The leukopoietic apparatus is also overactive with myelocytes and metamyelocytes predominating; even the promyelocytes may be increased in number. Mature neutrophils are reduced in number. Megakaryocytes may or may not be increased.

It has been found that whereas in normal persons glycolysis (that is, the digestion of sugar, or its utilization within the living organism) in blood containing not more than 100 mg. of dextrose per 100 c.c. is completed in about six hours, in the blood of patients with polycythemia the process was from two to three times as rapid, being completed in from one to four hours. Otherwise, blood chemistry shows no striking changes. Basal metabolism is elevated.

The important points in the laboratory diagnosis of polycythemia vera (the finding of a high red corpuscle count with high hemoglobin, and the finding of signs of increased bone marrow activity, polychromatophilia, nucleated red corpuscles, increased number of reticulocytes, leukocytosis with increase in the younger cells of the myeloid series, such as myelocytes and metamyelocytes, increase in the numbers of eosinophilic or basophilic leukocytes, thrombocytosis) justifiably arouse the suspicion of erythremia. Bone marrow examination may be of assistance.

Both the color index and volume index are low. The leukocytosis alluded to is around 15,000 to 16,000. There is a definite shift to the left, neutrophils forming about 75 per cent of the total differential picture.

The Treatment of Polycythemia

William Dameshek¹ gave a very excellent summary of the present concept concerning the treatment of polycythemia.

The treatment of polycythemia has passed through several successive stages since Osler first used benzol to reduce the excessive blood cell formation. Phenylhydrazine, a hemolytic poison, was introduced by Morawitz following its experimental use by J. H. Pratt. Although this new drug achieved wide popularity, its unpredictable nature and the severe hemolytic crises which often ensued made its use relatively hazardous. In some instances transfusions actu-

¹Dameshek, William: *Blood* 1: 256, 1946.

ally became necessary—a rather embarrassing situation. Other deleterious effects were on the circulation, the kidneys, and the liver. What is more, all the elements, including iron for enhancing further red cell formation, were retained within the body. For these reasons, the systematic use of multiple venesections to reduce the red cell mass and blood volume and induce a state of iron deficiency seemed far more physiologic. In Dameshek's experience, the removal of 500 c.c. of blood twice weekly for two to five weeks, depending upon the initial hematocrit and hemoglobin levels, has proved a satisfactory method. To maintain somewhat longer the resulting state of iron deficiency, it has been his practice to keep the patient on a diet low in iron. Red cell formation under these circumstances is only partially reduced, but hemoglobin and hematocrit levels remain low for periods of six to eighteen months, during which time the patient may be completely asymptomatic. Red cell levels during this induced remission gradually rise, so that the red cell count as an index of therapy is of little value. The best index is the hematocrit value, although the hemoglobin concentration alone may be used since this correlates fairly closely with the hematocrit level. With this method of therapy, patients go along for many years with very little more difficulty than do others in the older age group in which polycythemia occurs.

The use of x-ray therapy has, in Dameshek's hands, proved ineffective, either by heavy dosage to localized areas or by spray or generalized therapy. He has hesitated to use high voltage x-ray in these essentially normal individuals because of the possible dangers of radiation malignancy or leukemia. The introduction of radioactive phosphorus, as discussed by Erf,¹ makes therapy a good deal easier and is ordinarily productive of an excellent remission.

The treatment of patients with polycythemia vera with radioactive phosphorus (P^{32}) has become a well-established therapeutic approach. Lawrence and associates² report the nature and treatment of polycythemia vera in 263 patients. This series constitutes one of the largest groups studied for the longest time with radioactive phosphorus. These observers reflect the opinion generally held that irradiation therapy by this method is as safe, as simple, and as effective as any other known form of therapy. In our experience, radioactive phosphorus has been the treatment of choice, but in selected cases, such as those with considerably enlarged spleens, we have preferred radiation of the spleen by deep x-ray therapy. Isotope therapy has several advantages over deep x-ray therapy; namely, ease of administration, long remissions with single doses, absence of radiation sickness.

Thrombocytopenia of sufficient degree to cause bleeding may be an annoying complication but it not very frequent with standard dosages. In our experience, the hematologic effects of radiation, whether it be deep x-ray or isotope, are essentially the same. When radiation therapy is effective, the red blood cell count and hemoglobin diminish. As these values approach normal, symptoms generally improve.

We have used phlebotomy in patients with red blood cell counts over 6,500,000 per cu.mm. early in the program of therapy with isotope, since the effect of the therapy may not be evident before 3 to 6 weeks. The neces-

¹Erf, L. A.: *Blood* 1: 202-208, 1946.

²Lawrence, J. H., Berlin, N. I., and Huff, R. L.: *Medicine* 32: 323, 1953.

sity for further treatment has been based on the levels of the blood counts and symptoms. There does not appear to be any arbitrary level at which therapy is indicated. Block and co-workers¹ have treated some symptomatic patients with polycythemia vera with radioactive phosphorus even though their red cell counts have not been elevated. They have selected these cases on the basis of an extreme panhyperplasia and the absence of stainable iron in the bone marrow as seen in sections.

Other forms of therapy in polycythemia have been the use of nitrogen mustards by some. We feel that there are no apparent advantages of such therapy over irradiation, and there are obvious disadvantages, such as administration and toxic reactions.

There has always been and there still is a good deal of discussion concerning the relationship of polycythemia vera and leukemia or leukemoid states. The incidence of such relationships has always been very high and is generally considered a part of the natural history of polycythemia vera. There has been some understandable concern about the relationship of radiation therapy and such "complications" as leukemia. To date no strong evidence has been offered in this respect as related to the isotope therapy of polycythemia.

Symptomatic Polycythemia

In symptomatic polycythemia the relationship between red cell volume and plasma volume remains normal. There is no splenic tumor. The following forms of polycythemia have been described:

1. Concentration of the blood through abnormal perspiration or loss of fluid, such as we see in ulcer of the pylorus with frequent vomiting, in carcinoma of the esophagus, cholera, and trichinosis.
2. In cardiac and pulmonary diseases such as pneumothorax, apoplexy, emphysema, asthma, and bronchitis.
3. Toxic polycythemia due to carbon monoxide poisoning and acidosis.
4. So-called polycythemia hypertonica with atherosclerosis, hypertonia, and nephritis.
5. Splenic tuberculosis.
6. Splenomegalia.
7. Polycythemia of the newborn.
8. Compensatory polycythemia—seen in compensation in anemias and chlorosis, also in leukemias under roentgen ray treatment.
9. Hormone or internal secretory polycythemia.

Polycythemia hypertonica listed above has come under some scrutiny in the recent hematologic literature. Dameshek² apparently is speaking of this form of polycythemia under the title of "stress erythrocytosis." These individuals are usually men of the plethoric, obese, aggressive type with elevation of blood pressure. Acrocyanosis of the hands and feet may be pres-

¹Block, M., Bethard, W., and Jacobson, L. O.: Proc. Centra. Soc. Clin. Invest. 27: 16. 1954.

²Dameshek, W.: Blood 8: 282, 1953.

ent. Other than an elevation of the red blood count and hematocrit, all hematologic data are normal. Arterial oxygen saturation is normal, bone marrow studies are normal, and the red blood cell volume is normal. Plasma volume, however, is low. Lawrence and Berlin² emphasize the low plasma volume and suggest that it may be the result of long-continued psychic stress. These patients have such disturbances as peptic ulcers, coronary disease, and hypertension, a triad familiarly associated with psychosomatic disturbances. Dameshek stresses recognition of this group, chiefly to avoid the type of vigorous therapy traditionally given patients with polycythemia vera.

Polycythemia From High Elevations.—Bert³ described an increased hemoglobin in persons in the Andes from elevation of 4,400 meters. In the Himalayas, count of 8,300,000 has been described at an elevation of 6,000 meters. The natives of Tibet at an elevation of 4,866 meters show a red cell count of 7,600,000. At mountainous heights, the increase begins suddenly, becoming as high as 6,000,000 to 8,000,000 in twenty-four hours and reaching a definite height eight to fourteen days later. On returning to lower altitudes, the red cell count declines rapidly.

The cause of this elevation polycythemia has not been definitely determined. Some have claimed that it is due to the unequal distribution of red cells in the skin and in organs; others maintain that it is a vasomotor change. Meissen and his followers believe that it is a true increase in formation of red cells through lowered oxygen pressure.

AFFECTIONS OF THE WHITE CELLS

An increased number of leukocytes is called *leukocytosis*; a decreased number, *leukopenia*.

Leukocytosis

Variations may occur in the blood of the same individual even in health from day to day, or at different hours of the same day. These variations may be due to an increase in any one of the elements, that is, granulocytes, lymphocytes, or monocytes. In other words, there may be a granulocytic leukocytosis, lymphocytic leukocytosis, or monocytic leukocytosis. When there is damage to the tissue, the leukocytes mobilize from the blood to the site of injury and a discharge of these cells from the blood organs gives a general increase in the circulating blood. The purpose of the differential count is to tell what type of leukocytosis is at hand, whether of the granulocytic, lymphocytic, or monocytic variety. As a rule leukocytosis is usually granulocytic or neutrophilic. When we speak of neutrophilia we mean a neutrophilic leukocytosis. Neutrophilic leukocytosis occurs most frequently in acute infections, such as staphylococcus, streptococcus, etc. A similar leukocytosis occurs in pneumonia, whooping cough, scarlet fever, and some of the other infectious fevers.

Some authorities have spoken of digestive leukocytosis, that is, an increase in leukocytes during digestion, but this seems to be a misconception. There

²Lawrence, J. H., and Berlin, N. I.: *Yale J. Biol. & Med.* 24: 498, 1952.

³Bert, C.: *Acad. Sc., Paris*, 1902.

INFECTION-MILD GRADE



- 1. BLOOD PLATELETS
- 2. SEGMENTED NEUTROPHILE
- 3. EOSINOPHILE
- 4. LYMPHOCYTE
- 5. "STAB"
- 6. "STAB" WITH TWISTED NUCLEUS

NORMAL BLOOD PICTURE



- 1. BLOOD PLATELETS
- 2. SEGMENTED NEUTROPHILE
- 3. EOSINOPHILE
- 4. LYMPHOCYTE
- 5. MONOCYTE

NORMAL BLOOD PICTURE

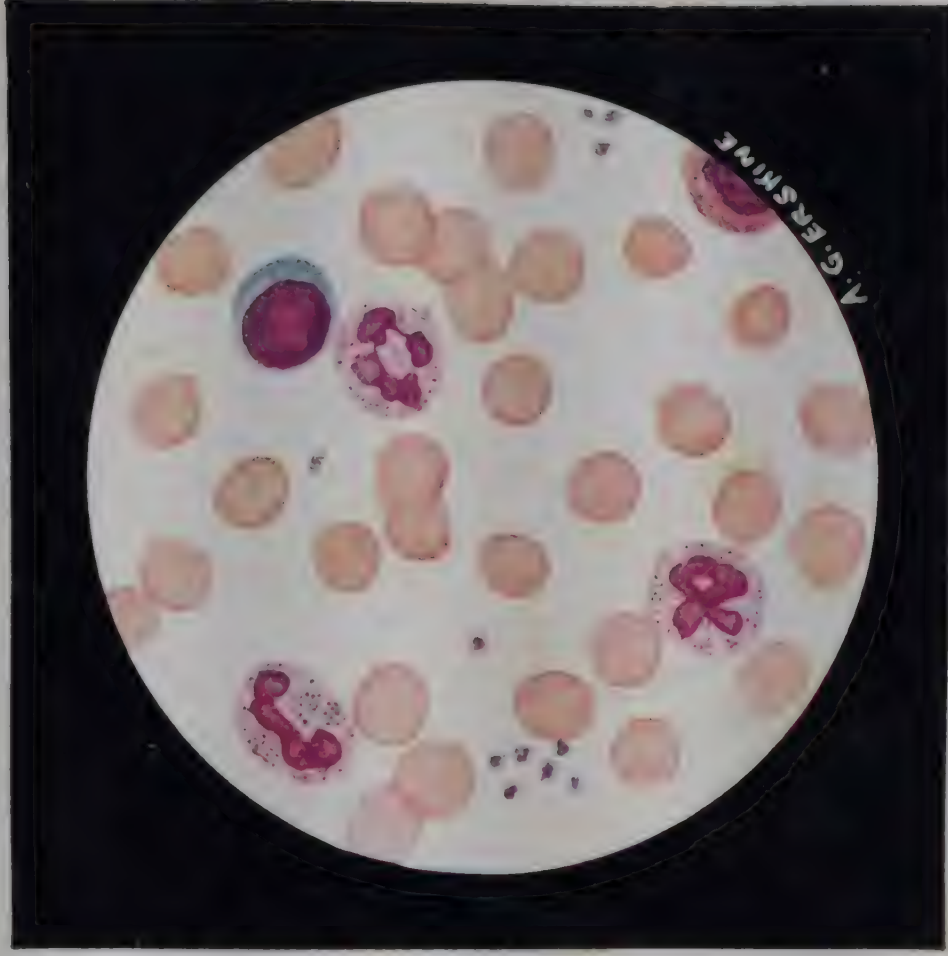
GIEMSA STAIN



X 950

INFECTION - MILD GRADE

GIEMSA STAIN



X 950

PLATE XIX.

seems to be a variation in the total white cell count during the day, usually a maximum of 9,000 to 10,000 in the afternoon and 5,000 to 8,000 in the morning. These variations occur quite independently of meals. Leukocytosis has been noted in pregnancy and during menstruation, and also as a result of muscular exercise or as a result of adrenal administration.

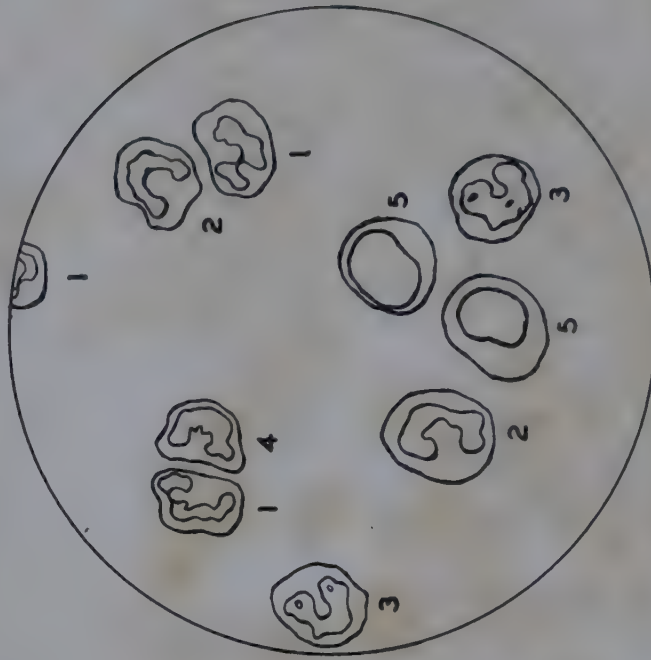
An increase in the number of eosinophiles, or eosinophilic leukocytosis, occurs in general in allergic states as in certain skin diseases, asthma, anaphylactic shock, and in infestations with animal parasites, such as the hookworm and the *Trichinella spiralis*. We have seen as many as 90 per cent eosinophiles in this latter condition accompanied by a marked increase in the total number of white cells.

In a general sense, the increase in the total number of leukocytes constitutes a normal protective reaction on the part of the blood. In disease, however, such as in leukemia, associated with increased number of leukocytes, this increase is a primary feature and not a reaction of defense in any sense. Leukemia is primarily a disease with a marked increase in the maturation and multiplication of white cells with appearance of abnormal forms due to factors at present unknown.

Explanation of the "Shift" of Schilling

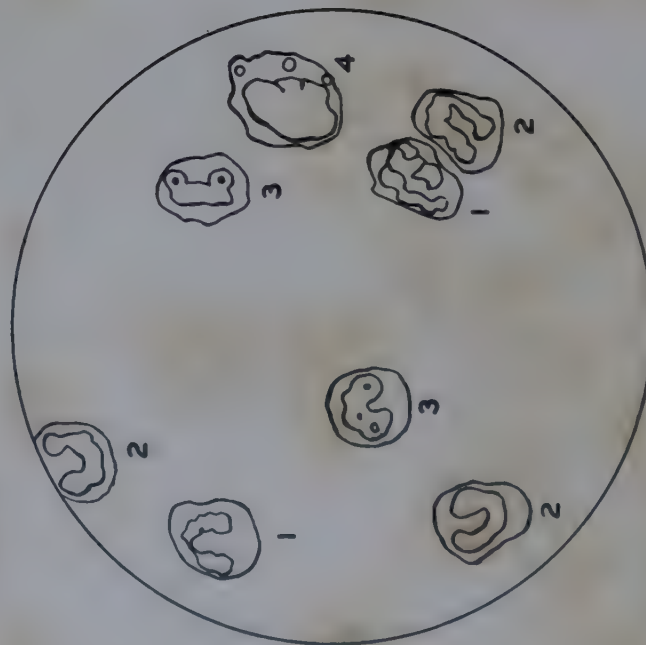
It is generally believed that the following order of cell development occurs in the myelocytic series: First, the myeloblast of Naegeli; next, the promyelocyte of Pappenheim; third, the myelocyte of Ehrlich; fourth, the juvenile of Schilling; fifth, the "stab" or band form of Schilling; sixth, the segmented cell. The last three are what were formerly termed the polymorphonuclear cells. We must assume that the bone marrow forms erythrocytes and granulocytes; that it is "irritable" or responsive or highly sensitive to an acute degree to chemotactic, humoral and vegetative-nervous insults; that it is the primary carrier of cellular defensive forces against toxic substances in the body, especially proteogenous antigens. It was Arneth who first called attention to the left shift of neutrophilic leukocytes as a very important development of histologic changes in connection with clinical diagnosis and prognosis. All the credit must be given to him for calling attention to these nuclear changes. His work, however, is not, strictly speaking, a practical procedure, nor do the other types of cells which he has described in his book (*Qualitative Blutlehre*) conform to the original idea of a shift of the neutrophiles. The total scheme comprises an observation of more than eighty cell species. His theory embodies a shift to the left of only juvenile cells. The Schilling count divides cells as follows: basophiles, eosinophiles, myelocytes, juveniles, "stabs" or rods, segments, lymphocytes, and monocytes. The normal quantities are: basophiles, 0-1; eosinophiles, 2-4; myelocytes, 0; juveniles, 0-1; "stabs," 3-5; segments, 58-66; lymphocytes, 21-30; monocytes, 4-8. The neutrophiles are myelocytes, juveniles, "stabs," and segments, or a total of 67. He has proved that in the presence of infection there is a definite change in the proportion of these cells. In the beginning, due to the toxic impression upon the bone marrow, there is an interruption in the development from "stabs" to segments, with a consequent increase in "stabs."

INFECTION—FATAL GRADE



- 1. SEGMENTED NEUTROPHILES
- 2. "STABS"
- 3. JUVENILES
- 4. DEGENERATED "STAB"
- 5. NEUTROPHILIC MYELOCYTES

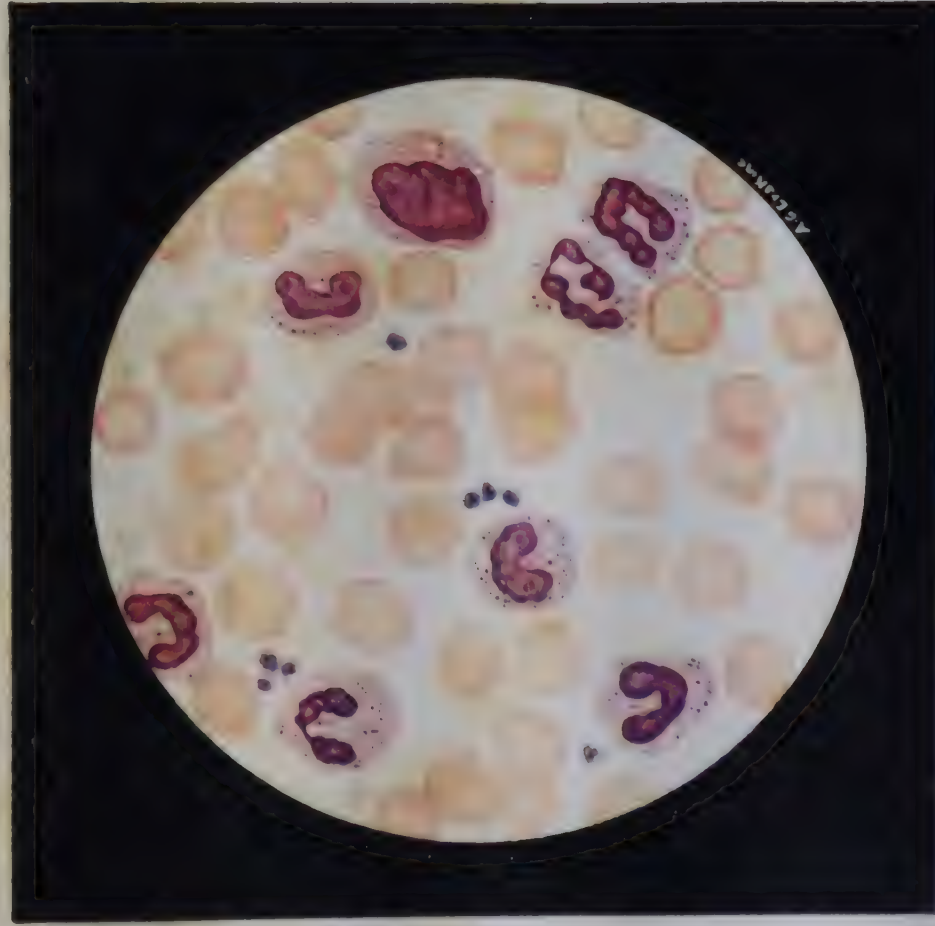
INFECTION—SEVERE GRADE



- 1. SEGMENTED NEUTROPHILES
- 2. "STABS"
- 3. JUVENILES
- 4. MONOCYTE

INFECTION - SEVERE GRADE

GIEMSA STAIN



X 950

INFECTION - FATAL GRADE

GIEMSA STAIN



X 950

PLATE XX.

To set forth the cells according to the Schilling method, construct a so-called hemogram as follows:

TOTAL NEUTROPHILES

	BASO- PHILE	EOSINO- PHILE	MYELO- CYTE	JUVE- NILE	STAB	SEGMENT- ED	LYMPHO- CYTE	MONO- CYTE
Normal %	(0-1)	(2-4)	(0)	(0-1)	(3-5)	(58-66)	(21-30)	(4-8)

Now draw a double line between the segments and the "stabs." If the neutrophilic cells to the left of this line increase, we call it a left shift. If the neutrophilic cells to the right increase, together with a decrease or absence of these cells on the left of the line, it is called a right shift. The true "right shift" shows numerous hypersegmented (6 or more lobes) neutrophiles.

A simple "degenerative blood picture" is a simple left shift in which the "stabs" increase in number. With a continued infection and with the defensive forces of the body acting intensively, under infection, this shift continues perhaps with the "stabs" remaining stationary, or increasing, but with the appearance of juvenile cells in the peripheral blood. At this time the appearance of juvenile cells indicates an irritation of bone marrow with regeneration. This is called a "regenerative blood picture." If the infection continues and the patient's resistance declines, the shift advances farther to the left. If improvement ensues, the shift declines and recedes to the right. A "leukemoid" shift is a shift in which the blood picture resembles leukemia; that is, a marked increase in myelocytes, juveniles, stabs, and segmented neutrophilic cells. In this type of shift, there are no eosinophiles or basophiles, and the number of lymphocytes and monocytes is very low; sometimes there are none. This is a serious blood picture.

Attention is also to be called to the change in number of the lymphocytes and monocytes during this infectious period. With a beginning shift to the left there is a coincident decline in lymphocytes. This decline continues until recuperation begins. With recuperation and retrogression of the shift toward the right, the lymphocytes increase. Manifestly a decline in lymphocytes indicates beginning infection, and increase in lymphocytes indicates resistance against infection. The neutrophilic regenerative shift implies a "battle" phase. Decline in lymphocytes indicates that the fortunes of war are with the invading organism; but with receding neutrophilia, a rising number of lymphocytes means a "phase of cure." At some stage in the infectious period when resistance overcomes invasion, the monocytes show a sudden rise. This occurs often even before the shift is materially changed to the right, and is what we term the "phase of defense." In the beginning of infections the eosinophiles decline and often totally disappear and reappear only when the shift turns about from left to right. This is termed "the dawn of convalescence." There is found, therefore, as a favorable prognosis, a return of eosinophiles, a shift changing from left to the right of neutrophiles, an increase in lymphocytes, and a marked increase in monocytes.

In chronic infectious conditions, a persistent high shift with low counts of eosinophiles and high counts of monocytes shows a chronic simultaneous

continuance of a phase of neutrophilic struggle and of monocytic defense. Thus the Schilling hemogram presents a kaleidoscopic view of infection in the blood picture which far surpasses in clinical import what we have

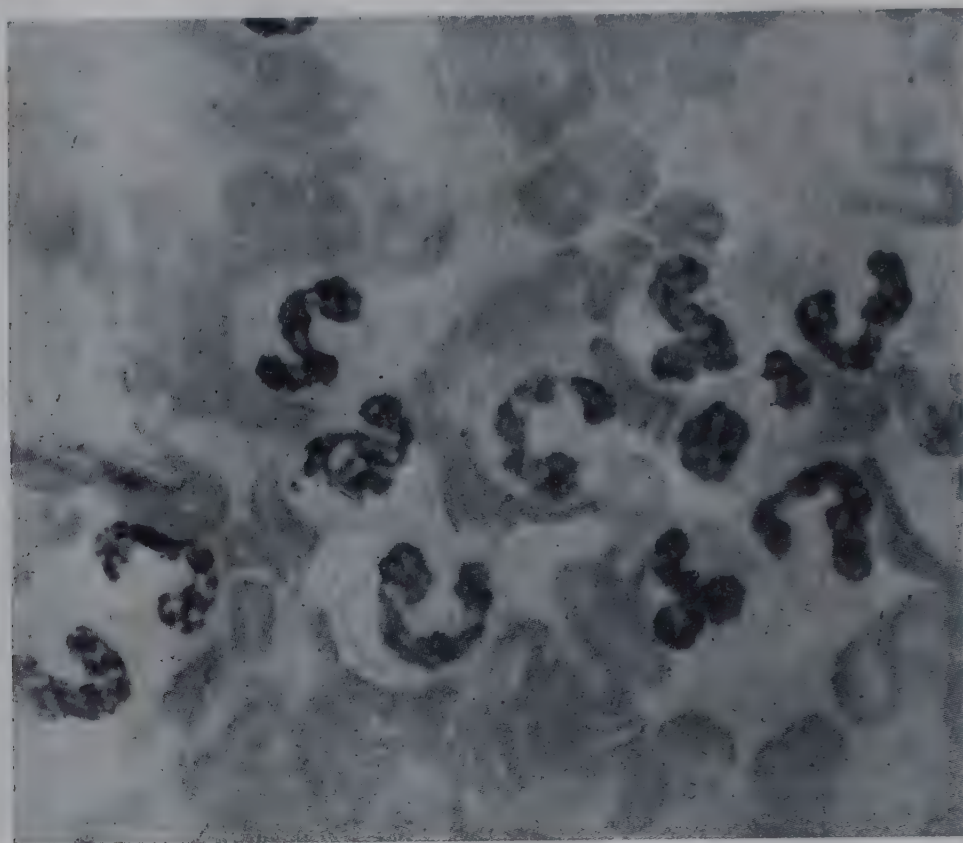


Fig. 196.—Tuberculosis, blood film, showing increase in "stabs" and segmented cells. ($\times 950$.)

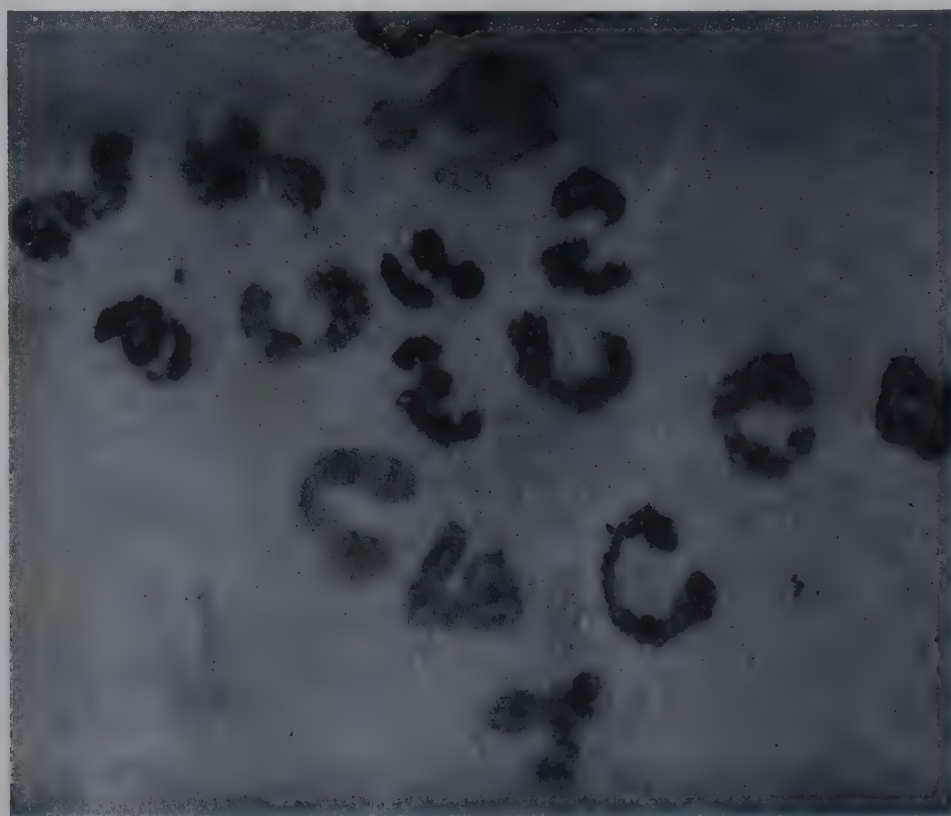


Fig. 197.—Acute infection, blood film, showing increase in juveniles and "stabs." ($\times 950$.)

been accustomed to see with the old Ehrlich differential count. We are enabled to note infection, to note susceptibility, and to note resistance. We are also able to note, very clearly, an increase in the severity of the infection and can predict, with certainty, impending death. It is in impending death

that we see the "flooding" or liberation picture where the bone marrow has finally been overcome and the peripheral blood shows almost intact cells ordinarily found only in the bone marrow.

Broadly speaking, the Schilling hemogram shows a biological reaction to disease, but not necessarily a specific picture for each infection. It is true that there are certain conditions of infection which give rather specific blood pictures, but it does not necessarily follow that a diagnosis of a specific infection can be made entirely by the blood picture. Certain conclusions can be drawn very accurately in many infections from the blood picture; for instance, in pneumonia, which is characterized by a hyperleukocytosis and a neutrophilia with regenerative shift. The blood findings in tuberculosis are highly interesting. With activity, there is a marked shift with decline in lymphocytes; with resistance, a recession of neutrophils to the right, and a marked increase in lymphocytes.

In typhoid fever there is a very interesting degenerative left shift and leukopenia. Particularly interesting is the aneosinophilia in typhoid fever. In typhus, on the contrary, we find what Schilling is pleased to term the "gay blood picture," which shows marked increase in stabs, quite a few juveniles, in other words, a regenerative blood picture, together with irritation forms and plasma cells, and an increase in monocytes. This "gay blood picture" is also seen in measles and German measles, but is less marked. It is to be noted that monocytes with increased white count are conspicuous in variola, but less marked in varicella, scarlet fever, and mumps.

It goes without saying that the Schiller differential count presupposes a knowledge of the exact appearance of the different types of blood cells. It is exceedingly important to recognize the difference between a juvenile, a "stab," and a segmented neutrophile. It is equally important not to confuse the lymphocytes with the monocytes. We have found that the work can best be done by the use of the Giemsa stain, and by rigidly adhering to the Schilling technic in general.

The **nuclear shift index** (Schilling) is used particularly when the number of neutrophils in the hemogram is low. The exclusive use of the index obscures the interpretation. The nuclear shift index is the relation of pathologic neutrophils (myelocytes + juveniles + "stabs") to segmented forms. Normally the index is 4:64, or 1:16.

Thus in a blood picture with no myelocytes, no juveniles, 2 "stabs," and only 18 segmented forms, the shift index is 1:9, which is a left shift.

Absolute Counts of Leukocytes

Absolute counts of each type of leukocyte are seldom made, but at times they are very valuable to determine whether there is overactivity or underactivity of certain hematopoietic systems where the differential count alone does not show this clearly. To obtain the absolute count of each type of white cell, multiply the patient's white count by the per cent of each cell as represented in the differential count.

Normally the average white count is 7,500 leukocytes. The normal differential count of Schilling is

Baso- philes 0-1	Eosino- philes 2-4	Myelo- cytes 0	Juveniles 0-1	Stabs 3-5	Segments 58-66	Lympho- cytes 21-30	Mono- cytes 4-8
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The absolute count in a normal individual, roughly, would be

Baso- philes 0 to 75	Eosino- philes 150 to 300	Myelo- cytes 0	Juveniles 0 to 75	Stabs 225 to 375	Segments 4,350 to 4,950	Lympho- cytes 1,575 to 2,250	Mono- cytes 300 to 600
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In the following case the absolute counts are given below the differential count.

White Count 2,400	Baso- philes —	Eosino- philes —	Myelo- cytes —	Juve- niles —	Stabs —	Seg- ments 35	Lympho- cytes 60	Mono- cytes 5
Absolute Count	—	—	—	—	—	840	1,440	120

At first glance it appears from the differential that there has been alteration in function of the lymphopoietic system which resulted in an increase of lymphocytes. From the absolute count this is clearly seen as only a relative change. The absolute number is slightly below the normal limits.

In pertussis, there is an absolute lymphocytosis. A leukocyte count of 25,000 or more with a great increase in lymphocytes manifestly shows involvement of the lymphocytic system; the white count increase is therefore due to increased production of lymphocytes

SPECIAL INTERPRETATION OF THE HEMOGRAM

(A) In Acute Infections.—

1. All blood pictures with beginning regenerative shifts and moderately high white counts are considered more favorable than those with degenerative shifts; nevertheless, rapidly increasing or very marked regenerative changes must be cautiously interpreted, especially if the white count decreases.
2. Marked signs of degeneration with decreasing white counts indicate a continuation of the infection.
3. Hyperregenerative and atypical hyperplastic blood pictures are serious symptoms that demand rapid removal of the cause, otherwise irreparable damage to hematopoiesis takes place and death will follow.
4. Constant aregenerative or aplastic blood pictures with low white counts are serious symptoms.
5. Approximately normal blood pictures and percentages must be seriously interpreted until sufficient time has elapsed to convince the *experienced* observer that the probable impending grave condition will not develop. The type of the hemogram, DETERMINED BY EXPERIENCE, must always form the basis of a general estimation in any given case.
6. Marked decrease of eosinophiles accompanied by rising leukocytosis, indicates an *aggravated* condition.
7. Total disappearance of eosinophiles (aneosinophilia*) with simultaneous marked lymphocytopenia is to be regarded as an *unfavorable* condition.
8. Constant presence of eosinophiles, especially if it parallels hyperleukocytoses, means a *favorable* situation.

*No eosinophiles in the thick drop.

9. Reappearance of eosinophiles (after absence from the thick drop) or increase in eosinophiles, with receding neutrophilia, with transition from lymphocytopenia to lymphocytosis, means a *very favorable* condition.
10. Moderately high white count, moderate differential change, without or with slight shift, and with eosinophiles, means a *favorable* condition.
11. The same picture in chronic conditions as an indication of a torpid cure shows a *doubtful* result.
12. Moderate or high white count with marked left shift, few eosinophiles, and slight decline in lymphocytes, indicates a condition not yet to be regarded as unfavorable.
13. Very high white count with marked left shift, with very young cells and marked neutrophilia, decrease of lymphocytes, and aneosinophilia, suggests a *doubtful* or *unfavorable* condition, especially if these findings become more pronounced.
14. Decreasing low white count, or leukopenia, with very high neutrophilia and beginning shift to the left, lymphocytopenia, and aneosinophilia, indicates a fatal prognosis.
15. Varying high white counts with constant hyperregenerative "leukemoid" or even irregular blood pictures mean a *very unfavorable* outcome.
16. Subnormal white counts with degenerative shift of neutrophiles, lymphocytosis, monocytosis, and presence of eosinophiles *per se* are *not unfavorable*.
17. Very low white counts with severe degenerative shift, neutropenia, and aneosinophilia, with only relative lymphocytosis and monocytosis, indicate an *unfavorable* state.
18. The retrogression of an existing shift (regenerative or degenerative) and of the neutrophilia or neutropenia, with approach to the normal count, with reappearance of the eosinophiles, is to be regarded as *always* favorable.
19. On the contrary, a blood picture showing a marked left shift deviating extensively from the normal count and accompanied by a decrease in or total disappearance of eosinophiles is *unfavorable*, and the more extensive the deviation from the normal, the more unfavorable the picture.
20. Marked decrease of lymphocytes in infections where there have been hyperleukocytoses of long duration shows a *complication*.
21. Sudden decrease of lymphocytes with increasing neutrophilia and shift, often with recurrent decreasing total white count ("fall of lymphocytes") is a *very serious condition*.
22. Parallelism between lymphocytosis and hyperleukocytosis is a *favorable symptom*.
23. Transition from neutrophilia to neutropenia, with receding shift, with increasing lymphocytosis, and eosinophilia is a *very favorable condition*.
24. Numerous younger or irritation lymphocytes with normal or abnormal white counts indicate active processes.
25. In acute infections with hyperleukocytoses, monocytosis is regarded as a *favorable symptom, receding only in more severe affections*.
26. In chronic infections, monocytosis indicates activity.

(B) In Anemias.—

1. Considerable polychromasia, or normoblasts without degenerative elements in the film, with increasing red count and color index near 1.0, indicates a *favorable* condition.
2. Very marked regeneration (numerous normoblasts and erythroblasts, considerable polychromasia) and a low red count is to be considered a *doubtful* picture.
3. A low red count, decreasing hemoglobin, and decreasing color index, without much polychromasia, with signs of degeneration (basophilic stippling, isolated normoblasts, nuclear debris, special forms), means an *unfavorable* picture.
4. A low red count, leukopenia, lymphocytosis, megalocytosis, and color index above 1.0 (megalocytic or pernicious anemia blood picture), means an *unfavorable* picture.
5. Constant low red and white counts without regeneration, followed by degeneration or aplasia, is distinctly a *very unfavorable* condition.

HEMOGRAMS TO ILLUSTRATE INTERPRETATIONS GIVEN ABOVE (ACUTE INFECTIONS)

EX-AMPLE NO.	LEUKOCYTES	B	E	M	J	ST	SEG	L	MONO	REMARKS
1a	12,300	-	-	-	4	16	51	25	4	Patient No. 1 (More favor- Patient No. 2 able than Pa- tient No. 2)
	3,800	-	-	-	-	32	38	24	6	
1b	12,300	-	-	-	4	16	51	25	4	Patient No. 1—Monday Tuesday. Change for the worse
	8,000	-	-	-	8	22	52	12	6	
2	7,800	-	1	-	-	10	61	25	3	Consecutive counts on same patient. Infection pro- gressing (unfavorable but not necessarily fatal)
	6,000	-	(+)	-	-	15	60	20	5	
	5,000	-	-	-	-	25	57	12	6	
3	23,000	-	(-)*	5	32½	28½	20½	8	4½	Hyper-regenerative shift; very unfavorable
4	2,500	-	-	-	-	-	18	75	7	Aregenerative shift; very unfavorable
6	8,600	½	3	-	-	6	58½	26½	5½	Consecutive counts on same patient; advancing infec- tion
	9,800	-	½	-	½	9	66	15	9	
	14,300	-	(-)	-	2½	14½	66	9	8	
7	16,000	-	(-)	½	15	30	41½	12½	½	Absence of eosinophiles; un- favorable
8 and 9	8,000	-	-	-	19	17	31	19	14	Nov. 23
	7,800	-	(+)	-	11	26	27	21	15	Nov. 24
	7,600	-	1	-	-	1	56	29	13	Dec. 14
	10,000	-	15	-	-	-	57	22	6	Jan. 5
10	11,900	-	4	-	-	10	65	14	7	Moderate infection; favor- able
12	15,000	-	½	-	½	21	58	17	3	Infection not yet regarded as unfavorable
13	23,000	-	(-)	5	33½	18½	20½	8	14½	Doubtful or unfavorable
14	10,000	-	(-)	-	2	8	81	7	2	Same patient
	4,800	-	(-)	-	3	10	83	1	3	Fatal picture
15	28,000	-	(-)	2	26	19	38	8	7	Unfavorable
16	4,000	-	(-)	-	-	20	30	40	10	If typhoid fever, picture is typical and not unfavor- able; if erysipelas or other acute infection, the pic- ture is very unfavorable (serious)
17	3,900	-	(-)	-	-	34	23	35	8	Very unfavorable in acute infections
18	13,800	-	(+)	-	2	19	50	21	8	Consecutive counts on the same patient. Favorable outcome
	12,000	-	1½	-	1	11	53½	23½	9½	
	10,000	-	1	-	-	4	59	25	11	
	10,000	-	5	-	-	6	59	20	10	
	8,000	-	2	-	-	2	67	20	10	
19	Very high	-	4	-	6	21	46	14	9	Consecutive counts on the same patient. Unfavor- able outcome
	Slightly lower	-	3½	-	9½	27	42	12	6	
	Slightly lower	½	½	-	10½	20½	39	21	8	
	Slightly lower	-	(-)	-	24	33	34	7	2	
	Extremely low	-	(-)	1	22	24	33	13	7	
20	18,000	-	1	-	2	20	50	20	7	Complication
	18,000	-	1	-	2	28	54	8	7	
	19,050	-	-	1	10	30	50	6	3	

*(-) No eosinophiles in the thick drop.

Problematic Hemograms in Infections

Blood pictures given below represent the type of picture expected in these diseases at the height of the infection. Manifestly complications occur, which will, of course, alter the blood picture. (See pages 791 ff. for specific hemograms.)

Scarlet Fever.—

Leukocytosis, neutrophilia, regenerative shift, slight eosinophilia, atypical lymphocytes and monocytes, plasma cells, Doehle's inclusion bodies. (The "gay" blood picture.)

Purulent Meningitis.—

Leukocytosis, neutrophilia, regenerative shift, hypoeosinophilia, lymphopenia.

Tuberculous Meningitis.—

Slight leukocytosis, eosinophilia, left shift (indefinite), neutrophilia, lymphopenia.

Pyelitis, Empyema, Puerperal Sepsis, General Septic Complications.—

Neutrophilia, usually leukocytosis (leukopenia if fatal), regenerative shift, lymphopenia, and eosinopenia or aneosinophilia; monocytes depend on the resistance of the individual.

Influenza, Dengue, etc.—

Leukopenia, slight stab shift, lymphocytosis, neutropenia, monocytosis.

Local Peritonitis.—

Moderately high white count, slight neutrophilia, slight shift, lymphopenia or normal lymphocyte count.

General Peritonitis.—

High leukocytosis, regenerative shift, neutrophilia, lymphopenia, eosinopenia or aneosinophilia.

Grippe, Uncomplicated.—

Leukopenia, neutropenia, slight stab shift (10%), lymphocytosis, monocytosis, presence of eosinophiles.

Grippe, Complicated with Pneumonia.—

Leukopenia becomes leukocytosis, degenerative shift becomes regenerative, lymphocytosis becomes lymphopenia, neutropenia becomes neutrophilia, and eosinophiles decline in number or disappear entirely.

Severe Mastoiditis.—

Leukocytosis, neutrophilia, regenerative shift, lymphopenia, eosinopenia. This condition sometimes shows only a stab shift if resistance is very low.

Measles.—

Leukopenia, regenerative shift, neutrophilia, lymphopenia, plasma cells (the "gay" blood picture).

Pertussis.—

Leukocytosis, lymphocytosis as high as 80%, neutropenia, left shift index.

General Infections.—

Leukocytosis (or leukopenia, if very unfavorable), neutrophilia, regenerative shift, lymphopenia, and hypoeosinophilia or aneosinophilia; monocytes depend on the resistance.

Polyarthritis Acuta.—

Leukocytosis, neutrophilia, right shift, or no shift, lymphopenia.

Erysipelas.—

Leukocytosis, neutrophilia, regenerative shift. Eosinophiles, monocytes, and lymphocytes depend on the resistance of the patient, although there is usually lymphopenia, and hypoeosinophilia or aneosinophilia.

Lobar Pneumonia and Senile Pneumonia.—

Leukocytosis, high grade regenerative shift, neutrophilia, lymphopenia, hypoeosinophilia or aneosinophilia, plasma cells.

Typhoid Fever, Uncomplicated.—

Leukopenia, neutropenia, degenerative shift (marked), aneosinophilia or hypoeosinophilia, lymphocytosis, and monocytosis.

Typhoid Fever with Septic Complications.—

Leukocytosis, regenerative shift, neutrophilia, lymphocytopenia, and absence or decrease of eosinophiles.

Typhus.—

Leukocytosis, neutrophilia, high-grade regenerative shift, atypical lymphocytes and monocytes, plasma cells (the "gay" blood picture).

Diphtheria.—

Leukocytosis, neutrophilia, regenerative shift, lymphopenia, eosinopenia or aneosinophilia.

Parotitis Epidemica.—

Neutropenia, leukopenia, lymphocytosis, eosinophilia, marked degenerative shift.

Rocky Mountain Spotted Fever.—

Leukopenia, or normal white count, neutropenia, unknown shift, monocytosis and lymphocytosis, with a marked anemia and the presence of marginal corpuscles.

Variola.—

Neutrophilia, regenerative shift, leukocytosis, plasma cells, the "gay" blood picture, lymphopenia.

Varicella.—

Neutropenia, leukopenia, unknown shift.

Poliomyelitis.—

Leukopenia, neutropenia, unknown shift.

Osteomyelitis.—

Hyperleukocytosis, neutrophilia, regenerative shift, hypoeosinophilia or aneosinophilia, lymphopenia.

Severe Streptococcus Infection, General.—

Marked leukocytosis (or leukopenia, as a serious finding), high neutrophilia, marked regenerative shift, lymphopenia, eosinopenia or aneosinophilia.

LEUKOPENIA

Leukopenia is a reduction in the number of leukocytes in the circulating blood below 4,500 per cu.mm. The leukopenic state has been reproduced experimentally and occurs clinically quite frequently. It has been the subject of much investigation.

Lawrence¹ lists five possibilities as playing a role in the pathogenesis of leukopenia:

1. Diminished manufacture of white blood cells. This may be the result of (a) simple inhibition, (b) maturation arrest, (c) aplasia of the bone marrow, and (d) infiltration of the bone marrow with foreign cells.

2. Increased elimination of white blood cells. This may occur when large quantities of cells are poured into infected areas, such as an empyema, or when large numbers of cells are lost through normal channels such as the gastrointestinal tract, the lungs, the spleen, or the liver.

3. Increased rate of destruction in the peripheral blood due either to abnormal white blood cells or to abnormal substances in the blood channels, which cause destruction of the white blood cells.

4. Redistribution of the white blood cells in the vascular channels, such as occurs with foreign proteins.

5. Redistribution of the white blood cells in the body as a whole, as is seen at times in leukopenic phases of leukemia.

Although this summary of the causes of leukopenia is apparently all-inclusive, it is very difficult to fortify all these conditions with experimental or clinical facts.

It is thought that virus infections, typhoid fever, malaria, and overwhelming infections can be cited as examples of simple inhibition; nevertheless, there

¹Lawrence, J. S.: J. A. M. A. 110: 478, 1941.

is a possibility that other mechanisms enter into the causation of leukopenia in these conditions. Virus infections, such as influenza, dengue, viral hepatitis, viral pneumonia, acute poliomyelitis, vaccinia, are almost always accompanied by leukopenia. It is rather interesting that such a large number of virus infections is associated with a low white blood count, which is in sharp contrast with the leukocytosis which occurs in most bacterial infections. It might be worth while philosophizing that in viral infections, leukocytes are not a predominant defense mechanism, and are therefore not called upon to handle the infection. Typhoid and malaria (after the attack) are given as examples of simple inhibition, but it is rather interesting that both of these diseases are associated with splenomegaly, a condition which is associated with leukopenic states, perhaps due to some form of "hypersplenism."

Maturation arrest of cells of the myelocytic series is most frequently associated with agranulocytosis, due to drug intoxication, such as the sulfonamides. In these instances, some hypersensitivity phenomenon has been implicated as the *modus operandi* of the maturation arrest phenomenon.

Although leukopenia may be seen at times in aplasia of the bone marrow, it is rather surprising how infrequently this phenomenon is associated with rather marked aplasia of the bone marrow in various clinical conditions. Such aplasia may be due to idiopathic states or may result from therapy which destroys the bone marrow. Such therapy includes radiation, both in the form of x-ray and radioactive isotopes, and may be associated as well with severe nutritional deficiencies.

Infiltration of the bone marrow with tumor cells of various kinds includes metastatic carcinoma, multiple myeloma, and other diseases, but a simple explanation is not forthcoming for the leukopenia associated with such states. The relatively simple explanation of overcrowding by tumor cells is hardly acceptable. The author (H. A.) has observed numerous cases of widespread invasion of the bone marrow by carcinoma and in sections of these marrows has seen very active myelopoiesis alongside invading tumor.

When we speak of diminished manufacture of white blood cells within the bone marrow as a cause of leukopenia, we must also take into account the delivery of cells into the peripheral blood. This is a subject about which, at the present time, we know practically nothing. There are numerous examples of bone marrow overcrowded with white blood cells and with a peripheral leukopenia and vice versa.

Palmer and associates¹ have been able to reproduce in rats a disorder closely resembling the clinical syndrome in man to which the term "secondary hypersplenism" has been applied. This syndrome, produced by the injection of methyl cellulose in rats, presents a picture characterized by splenomegaly, hyperplasia of the bone marrow, and leukopenia, among other things. In their animals, if splenectomy were performed prior to the injection of the methyl cellulose, no leukopenia occurred; in fact, a splenectomy type of leukocytosis appeared; whereas in animals injected with methyl cellulose and developing the full syndrome, leukopenia was evident. They point out the

¹Palmer, J. G., Eichwald, E. J., Cartwright, G. E., and Wintrobe, M. M.: *Blood* 8: 72, 1952.

prophylactic role of the splenectomy in their animals, and also state that infiltration of the bone marrow by methyl cellulose-containing macrophages is not the explanation for the pathogenesis of these changes. They particularly point out that pancytopenia failed to develop in splenectomized animals, even though there was an equal degree of infiltration of the bone marrow by the methyl cellulose, and the bone marrows of the animals given the methyl cellulose were characterized by hyperplasia of the blood-forming tissue.

No discussion of the subject of leukopenia could be complete without invoking the present interest in immunologic processes as a cause of anemia, thrombopenia, and leukopenia. Well-documented experimental and clinical studies have been numerous in the literature concerning the role of immunologic abnormalities relating to the red blood cells, hemoglobin, and the platelets. However, fewer reports are present concerning leukocytes. Kissmeyer-Nielsen¹ carried out transfusion experiments to prove the presence of a leukopenic factor in the blood of patients with leukopenia, and he believed that he could demonstrate such relationship.

In speaking of the leukopenic phases of leukemia, most observers are referring mainly to granulopenia or neutropenia. In acute agranulocytosis, unquestionably the disappearance of the granulocytes is due to the failure of the myeloblasts or the myelocytes to mature into adult cells. It has been found that the bone marrow varies according to the stage of the disease, but at times myeloblasts may be the only representatives of the granulocytic series.

Only a few minutes are required to cause the disappearance of granulocytes from the blood after a small dose of amidopyrine in some patients. Vejens, in Norway, found that certain hydrophile colloids, like globulin, which produce leukopenia when injected intravenously, do so by causing the white cells to take up a marginal position in the venules of internal organs such as the liver, spleen, and omentum. This is really a type of "leukocytic shock" because the leukocytes are temporarily out of the circulation. This may be caused by gelatin, globulin, and fibrinogen.

Piney² called attention to chronic neutropenia which falls in the diminished manufacture group. Here the only clinical syndrome is apparently causeless fatigue or lassitude, with a granulopenia of less than 200 cells per cu.mm. In these cases, treatment, including pentnucleotide, fails to influence this neutropenia, although Piney's patient recovered after receiving Hepatex T.

With respect to the fifth possibility, redistribution in the body as a whole, the mechanism that is responsible for the distribution of the white blood cells between the tissues and the vascular channels is unknown. Lawrence gives as the best illustration of this the so-called aleukemic leukemia. In this condition there may be leukopenia and yet the various tissues in the body may be filled up with white blood cells. That this may occur in other conditions is a distinct possibility.

Many of the facts relating to the cause of leukopenia have yet to be developed.

¹Kissmeyer-Nielsen, F.: *Acta haemat.* 9: 337, 1953.

²Piney, A.: *Lancet* 1: 348, 1941.

BLOOD PICTURE IN VARIOUS INFECTIOUS DISEASES

Typhoid Fever

Typhoid fever is characterized by leukopenia down to 3,000 to 5,000 per cu.mm., and a left shift. Eosinophiles disappear at the beginning of typhoid fever. They do not reappear until the third week of the infection.

We may read from the hemogram certain definite prognostic facts in typhoid fever as follows:

1. A very few eosinophiles in the first stage speaks for a mild infection. The reappearance of the eosinophiles in the third stage of typhoid fever is a good prognostic factor. A relatively high neutrophile percentage is found in the prodromal stages; as the disease becomes more pronounced there is neutropenia plus lymphocytosis. A supervening neutrophilia is indicative of a possible complication, especially if there is a regenerative shift. A return of lymphocytes or a regression of the shift in the third stage is a favorable symptom.

2. Unfavorable symptoms are a continued leukopenia and an increasing left shift.

The mechanics of the lowered white count in typhoid fever is undoubtedly a toxic insufficiency of the bone marrow. See page 788. Studies of the bone marrow in this disease show very few myelocytes and many myeloblasts.

Typical typhoid fever counts are as follows:

White Cells 3,500. Slight polychromasia.

Bas.	Eos.	Myel.	Juv.	Stab		Segs.	Lymphs.	Monos.
0	0	0	0	18		39	35	8

White Cells 8,450. Red Cells 3,360,000. Hb. 65%. CI 0.9.

Bas.	Eos.	Myel.	Juv.	Stab		Segs.	Lymphs.	Monos.
0	0	0	4	48		15	33	0

White Cells 6,650.

Bas.	Eos.	Myel.	Juv.	Stab		Segs.	Lymphs.	Monos.
0	0	0	12	32		23	32	1

White Cells 11,800.

Bas.	Eos.	Myel.	Juv.	Stab		Segs.	Lymphs.	Monos.
0	0	2	7	43		23	24	1

Paratyphoid A and B Infections

Paratyphoid A and B cases show a reduction, possibly to one-half million, of red cells with a lowered hemoglobin content and therefore a lowered color index. The leukopenia is not as pronounced as it is in typhoid fever. The left shift is about the same.

Typhus

In typhus the hemogram is as follows:

Total white count 8,000.

Bas.	Eos.	Myel.	Juv.	Stab		Segs.	Lymphs.	Monos.
0	0	0	5	16		46	27	6

There are many plasma cells. Schilling calls this picture the "gay" or the "colorful" blood picture.

Naegeli is inclined to think of a slight leukocytosis in typhus. He cites seventeen cases by Slatineano with 14,000 leukocytes, and thirteen cases by Cazeneuve with from 10,000 to 30,000 leukocytes. Rothacker does not think that the leukocyte count is ever higher than 14,000.

The diagnostic symptoms in the blood picture of this disease are the "gay" blood picture with a left regenerative shift. Eosinophiles do not disappear quite as rapidly and uniformly as they do in typhoid fever. Cazeneuve places considerable diagnostic importance upon the appearance of myelocytes (2 to 12 per cent) in typhus.

Most authorities speak of a marked decline in the number of red cells in this disease, accompanied by a hemoglobin drop, sometimes down to 52 per cent. Dychno¹ describes red cell resistance figures up to 0.32 to 0.36 per cent.

Malaria

The general blood changes in malaria are as follows: anemia is always present. It is caused both by direct destruction of erythrocytes due to the development of the parasites within them and also by toxic influences. There is oligocytosis, a lowered hemoglobin content, and a lowered color index. Polychromasia is usually marked, and coarse basophilic punctation is a frequent blood finding. In cases with severe anemia normoblasts are found. There may be nuclear spheres, marginal granules, and other nonspecific indicators of anemia. Parasites have been observed in polychromatic and stippled erythrocytes, and even in normoblasts.

Malaria pigment may frequently be found in phagocytes, usually in large monocytes (pigmentophages), although we have seen them in all four types of neutrophilic cells. This pigment is yellow or brown, and light refracting. It is usually arranged in characteristic clumps. It originates from devoured or phagocytized parasites and is important in detecting malarial infection when no parasites are found. It is demonstrable not only in blood spreads, but also in thick drop preparations.

During the attack, there is irritation leukocytosis with marked regenerative shift, neutrophilia, and decrease in the number of eosinophiles. *During reduction of fever and shortly following this*, there is leukopenia, marked monocytosis and relative lymphocytosis, with slight eosinophilia. In severe relapsing cases there is a continuous "stab" shift. In *chronic cases*, low white counts, monocytosis and lymphocytosis are found. The white count rises rapidly, but only temporarily, shortly after an open or latent attack. In this condition parasites are scarce. If there is a continued shift with monocytosis after treatment, it is thought to indicate recurrence. In clinically suspected malaria where no parasites are found in the blood, one is led to the diagnosis of malaria if polychromasia is present as two or three plus in the thick drop, if basophilic punctation is found, and if lymphocytes and monocytes are markedly increased, especially if quinine has already been administered.

¹Dychno: *Ztschr. Hyg.* 101: 203, 1923. Quoted by Naegeli, L. C.

All varieties of malaria show similar blood pictures, but acute *falciparum* malaria may show a higher neutrophilia with a regenerative shift to myelocytes.

In parasite-negative cases, the administration of 0.5 to 1 c.c. of 1 per cent Adrenalin may aid in the demonstration of the parasites. Other methods that have been used are repeated ultraviolet ray exposures, severe physiotherapy with alternating hot and cold douches. Thick drop should be examined repeatedly at intervals of several hours.

In blackwater fever there is a marked decrease in the number of erythrocytes and in hemoglobin content. This is soon followed, with improvement, by a very marked regenerative normoblastic blood picture. Upon continuation or onset of new attacks, megaloblastic or aregenerative (reverse) blood pictures occur. This is an unfavorable symptom. Deaths have occurred in blackwater fever due to suppression of erythropoiesis. Parasites usually disappear temporarily during an attack of blackwater fever.

Falciparum Malaria

In tropical malaria, there may be both a hyperleukocytosis and a left shift. The typical hemogram of **falciparum malaria** is as follows:

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	0	0	18	32		20	18	12

In the thick drop there are marked polychromasia and basophilic punctation and many ring forms of *Plasmodium falciparum*.

In this disease there is a severe hyperleukocytosis with a comparatively high lymphocytosis and an increase in monocytes. There are no eosinophiles and there is a marked toxic polychromasia.

Influenza

Influenza is characterized by a leukopenia with a left shift. A typical hemogram is as follows:

White Count 4,000.								
Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	0	0	0	15		58	23	4

Tuberculosis

Beginning tuberculosis shows an increased total white count and distinct left shift.

The erythrocyte sedimentation test, while not specific for tuberculosis, is useful in following the progress of the disease. With the Wintrobe tube, the following designations have been introduced by Doan, Wiseman, Mercer, and Miller.¹ Designate arbitrarily the normal rate as +. Deviations from the normal are designated ++, +++, or +++, depending upon the sedimentation velocity of the red blood cells as evidenced by the type of curve obtained during the first hour, a fall approaching the vertical being designated as +.

¹Doan, C. A., Wiseman, B. K., Mercer, S. T., and Miller, M. D.: Ohio S. M. J. 28: 333, 1932.

Severely ill patients all show a maximum fall in one hour. With general clinical improvement and regressive lesions the sedimentation rate returns to normal.

The red cells show a slight reduction in total numbers in tuberculosis, except in the terminal stages.

The count of the white blood cells is very important in tuberculosis. The discovery by Sabin, Doan, and Cunningham² of the relationship which exists between the monocyte of the circulating blood and the epithelioid cell of the tubercle forms the basis for the increased interest in the cellular mechanism of this disease. Cunningham, Sabin, et al.³ first showed the importance of the monocyte-lymphocyte ratio as obtained from differential counts of the circulating blood. Progressive tuberculous lesions showed a gradually increasing monocyte and a decreasing lymphocyte count, while regressing lesions were accompanied by a reversal of these relationships, that is, by a lowering of the monocyte-lymphocyte index in the blood (normal is 0.33). Qualitative changes in the monocytes, with the appearance of epithelioid cells in the peripheral blood, are quite as significant as the quantitative changes just mentioned. Medlar⁴ has emphasized the importance of the neutrophilic leukocytes in appraising the incipency and extent of caseation and cavitation in tuberculous foci. Wiseman⁵ re-evaluated certain qualitative changes in lymphocytes with reference to age and maturity, which when applied to the study of these cells in tuberculous cases further aids in the analysis and prognosis of the disease. Wiseman found the normal average lymphocyte formula to be 5 per cent young basophilic types, 45 per cent mature moderately basophilic forms, and 50 per cent old, faintly basophilic lymphocytes. In tuberculosis, the increase in young, immature forms denotes an additional strain placed on the lymphatic tissues. It has been recognized for a long time that resistance to tuberculosis is associated with a relative lymphocytosis, but it is essential that the lymphocytes be relatively mature to serve their function. Thus, in known tuberculosis patients, if the total lymphocyte count is relatively low, with a degenerative rather than a regenerative "shift to the left," the prognosis is grave, even though the monocytic-lymphocytic index may not be markedly increased.

In tuberculosis, there is a shift of the neutrophilic leukocytes with an increase in the "stabs." With increasing severity, there is a regenerative blood picture. The commonest type, however, is the degenerative picture. Progressive cases show a falling lymphocytosis. Cases doing well show normal lymphocytes, or an increase. Eosinophiles are usually present and their presence or absence is of little significance. The same is true of basophiles. Allusion has already been made to the ratios between the monocytes and lymphocytes.

²Sabin, F. R., Doan, C. A., Cunningham, R. S.: *Carnegie Inst. of Wash., Pub. No. 82, Contrib. to Embryol.* 14: 125, 1925.

³Cunningham, R. S., Sabin, F. R., Sugiyama, S., and Kindwall, J. A.: *Bull. Johns Hopkins Hosp.* 37: 231, 1925.

⁴Medlar, E. M.: *Am. Rev. Tuberc.* 20: 312, 1929.

⁵Wiseman, B. K.: *J. Exper. Med.* 53: 499, 1931.

Bredeck⁶ in the study of fifty-nine cases of tuberculosis, with reference to the Schilling differential count, showed that all definitely active cases of pulmonary tuberculosis have a positive infectious blood picture. Three cases, in which a diagnosis of tuberculosis was made by other physicians some time ago, showed no evidence of tuberculosis except by x-ray, but these three had negative infectious blood pictures.

Bredeck instituted a method for utilizing the tuberculin test in connection with the Schilling blood count as a guide, with a diagnosis of tuberculosis established, in determining the progress of the disease. Tuberculin causes a specific focal reaction with characteristic blood changes. In short, it is the best agent for inducing focal activity. His method is to make a differential blood picture and then give an injection of tuberculin, 10 mg. After this injection, if the patient is tuberculous, there will be a characteristic change in the blood picture, decrease in the lymphocytes and a marked increase in the "stab" cells, and an increase in monocytes. Changes in the blood differential count after tuberculin occur before the fever reaction, and with smaller doses of tuberculin than those that induce fever. The Schilling count, together with the subcutaneous tuberculin test, constitutes the most delicate and most accurate methods in the diagnosis of early manifestations and induced focal activity. A shifting of the neutrophilic leukocytes to the left in the Schilling blood differential count is a constant finding in clinically active tuberculosis. An increase in lymphocytes indicates healing and a diminution indicates progression of the disease. High lymphocyte counts, with increase in monocytes and with little or no change in neutrophils, indicate healing. A drop in lymphocytes and an increase in monocytes, with an increase in the shifting to the left of the neutrophils, indicate active progression of the disease. The prognosis of tuberculosis depends upon the severity of the shifting of the neutrophils to the left and degree of changes in the number of lymphocytes. This, together with the Wiseman count of the lymphocytes, constitutes an admirable method for estimating the future of these tuberculosis cases.

Typical hemograms follow:

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	1	0	0	6		54	27	12

Chronic tuberculosis with cavities shows:

White Count 10,000.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	2	0	0	9		46	34	9

A severe picture of active tuberculosis, with serious prognosis, follows:

White Count 20,000.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	1	0	0	20		51	19	9

Advanced tuberculosis, as follows:

White Count 22,000.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	0	0	7	38		17	14	24

⁶Bredeck, J. F.: *Am. Rev. Tuberc.* 20: 52, 1929.

Pulmonary tuberculosis, as follows:

White Count 14,800. Red Count 4,620,000. Hb. 88%									
Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.	
0	0	0	0	10		69	19	2	

Pulmonary tuberculosis, as follows: (Fatal Case)

White Count 5,900. Red Count 4,300,000. Hb. 80%. C.I. 0.9.									
Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.	
0	0	0	0	20		73	6	1	

Bone tuberculosis:

White Count 10,100. Red Count 4,480,000. Hb. 85%.									
Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.	
0	0	0	0	2		67	29	2	

Testicle tuberculosis:

White Count 11,650. Red Count 4,850,000. Hb. 90%.									
Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.	
0	2	0	0	5		70	21	2	

Croupous Pneumonia

In croupous pneumonia there is a marked hyperleukocytosis, together with a very severe left shift. The total counts may exceed 35,000, especially in children, when it may be 50,000. This is a marked neutrophilic leukocytosis with a left shift. Occasionally myelocytes may be found during the height of the disease. The leukocytosis occurs very soon after the beginning chill and remains until the crisis occurs. With the crisis, there is a return of the eosinophiles and the appearance of monocytes—the so-called “dawn of convalescence.” One is accustomed to think of a severe hyperleukocytosis in croupous pneumonia as a favorable symptom and the absence of a leukocytosis as a very unfavorable symptom. If leukopenia is found in croupous pneumonia, the prognosis is very grave. There is very little change in the red cell picture in croupous pneumonia, and only a very small reduction in the erythrocytes and hemoglobin. Blood platelets are increased and the sedimentation rate is markedly heightened.

The following hemogram is from a man 25 years of age, with pulse 110, respiration to 40, temperature 104° on the fourth day of the disease.

White Count 12,575. Red Count 4,865,000.									
Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.	
0	0	0	15	51		27½	10½	1	

Another interesting series of hemograms follows from a case of left-upper lobar pneumonia. Note that on November 21, the day of the first hemogram, the patient was at the height of his process, having a temperature of 104° F. The crisis occurred on November 25. At that time his temperature dropped to 99.4°, becoming absolutely normal on December 3. Note hemograms of November 25 and December 3.

Date	Total Leukocytes	Bas.	Eos.	Myel.	Juv.	Stabs	Seg.	Lymph.	Monos.
11-21-34	23,500	0	0	0	8	38	47	5	2
11-25-34	13,775	0	3	0	0	15	47	22	13
12-3-34	10,950	0	0	0	0	6	59	31	4
12-7-34	9,313	0	1	0	0	5	34	51	9

The effect of the administration of sulfathiazole on the blood picture in lobar pneumonia is well seen in the following case from our service at the Christian Hospital, St. Louis. Note the lowering of the leukocyte count, together with a more favorable shift of the neutrophils, and the return of the eosinophiles. Monocytes are increased, forecasting recovery.

The patient, a 58-year-old white woman, entered the hospital on Jan. 3, 1942. Several days before entry she became ill, with chills, coughing, and finally expectoration of rusty sputum, and pain in the left chest. There were no cardiac or gastrointestinal or genitourinary complaints. Patient was slightly undernourished, coughing, febrile, and looked very ill. Abdomen: soft; no tenderness or masses, or palpable viscera. Heart sounds were of good quality. Blood pressure: 115/70. Lungs: Bronchial breathing, increased fremitus, flatness of left lower lobe, with moist râles. Also moist râles and bronchovesicular breathing over spots in the right middle lobe and right base. Kidneys not tender, not palpable. Reflexes normal. Very slight edema of legs.

HOS- PI- TAL DAY	SULFA- THIAZOLE	LEU- KO- CYTES	ERYTHRO- CYTES	HGB. SAHLI	C.I.	B.	E.	M.	J.	ST.	SEG.	L.	MONO.	BLOOD SUL- FATHIAZOLE
1	30 gr. twice	32,050	4,240,000	84%	1.0	-	-	-	2	16	62	15	5	
2	15 gr. every 4 hours	48,100	4,240,000	84%	1.0	-	-		4	11	67	11	7	11 mg.
3	15 gr. every 6 hours													1.7 mg.
4	15 gr. three times daily	25,450	4,260,000	84%	1.0	-	-	-	2	7	71	18	2	(Coarse toxic granules; 1 plasma cell)
6		14,750	4,210,000	87%	1.0	-	1	2	6	7	67	12	5	
9	Discon- tinued	13,400	5,070,000	87%	0.87	-	1	-	1	2	57	29	10	
14	15 gr. 4 times daily 15 gr. every 4 hours	12,000	4,390,000	79%	0.92	-	-	-	-	2	81	10	7	
16	15 gr. every 6 hours	11,000	3,910,000	76%	0.9	1	1	-	-	-	67	25	6	
19	15 gr. once a day	9,950	4,440,000	79%	0.9	1	2	-	-	-	52	39	6	Amount too minute to estimate
26	Dismissed													

The sputum was of tenacious and rusty character. Pneumococci Type I were present. Pulse was 120; temperature, 103.6° F.; respiration, 24. Diagnosis: pneumonia. Sulfathiazole, 30 grains, was administered twice on the first day, then 15 grains every four hours there-

after. Oxygen was administered through nasal catheter. Treatment was continued throughout the second day. Patient seemed in a stupor. Pulse thready. Urinated involuntarily. Fingertips cyanotic.

The accompanying series of blood pictures was obtained. (Patient was discharged on the twenty-sixth day.)

The following case from our service at the Christian Hospital, St. Louis, Mo., is presented through the courtesy of Dr. H. A. Klein.

Patient, A. G., a well-developed male, entered the hospital on May 11, 1945, with history of chills and fever and chest pains. Râles in entire right lung. History of illness, two weeks. Left humerus broken; since then weak cough. Diagnosis: Lobar pneumonia, right, middle, and upper lobes.

	W.B.C.	R.B.C.	HB.	C.I.	B.	E.	M.	J.	ST.	SEG.	L.	MON.	
5/11/45	6,200	3,500,000	75%	1.0	-	-	-	-	2	88	10	-	Sulfadiazine xv gr. every 4 hr.
5/12/45													500 c.c. whole blood transfusion
5/13/45													500 c.c. plasma; discontinued sulfadiazine
5/14/45	7,500	3,670,000	79%	1.0	-	2	-	-	8	70	20	-	Penicillin 20,000 units every 4 hr.
5/16/45	7,900	3,750,000	80%	1.0	-	-	-	-	6	74	20	-	Penicillin discontinued; 500 c.c. whole blood
5/19/45	9,200	3,900,000	75%	0.9	-	-	-	-	6	84	4	6	
6/11/45	8,700	3,860,000	80%	1.0	-	-	-	-	4	70	26	-	
6/18/45	8,500	3,662,500	75%	1.0	-	2	-	-	6	70	20	2	

Patient was dismissed on June 27, 1945.

Bronchopneumonia

The leukocytosis in bronchopneumonia is not nearly as high as it is in croupous pneumonia, seldom over 15,000. In bronchopneumonia due to influenza, as already stated, the picture is a leukopenia and there is a left shift.

Virus or Atypical Pneumonia

In contradistinction to bronchopneumonia, virus pneumonias have leukopenia with a left shift. However, there generally is present a moderate lymphocytosis. Owrens, Kelley, and Agress¹ showed that in some cases there may occur a very marked absolute and relative lymphocytosis associated with a leukocytosis up to 20,000. During this period, such cases may be confused with acute infectious mononucleosis. However, the pathologic lymphocytes of acute infectious mononucleosis either were absent or were present in only small numbers. The heterophile antibody test was negative.

Tularemia

The blood picture in tularemia is a neutrophilia with a left shift. Hemograms of two cases reported by the author² are as shown in Tables 60 and 61.

¹Owrens, M. H., Kelley, R. W., and Agress, H.: Med. Bull. North African Theatre of Operations 2: 56, 1944.
²Gradwohl, R. B. H.: J. Missouri M. A., pp. 123-124, 1933.

TABLE 60.—BLOOD PICTURE IN CASE 1

TULAREMIA	TEMPERATURE	RED BLOOD CELLS	WHITE BLOOD CELLS	BASOPHILES	EOSINOPHILES	MYELOCYTES	JUVENILES	STABS	SEGMENTED LYMPHOCYTES	MONOCYTES	
November 21 -----	100.4	4,765,000	19,550	1	0	0	6	53	23	12	5
						82					
November 22. Agglutination for <i>Pasteurella tularensis</i> positive	100	4,755,000	17,000	1	1	0	4	48	29	13	4
						81					
November 23 -----	100.4	5,000,000	15,800	0	1	0	4	32	26	21	16
						62					
December 4 -----	99		20,850	2	0	0	1	42	32	19	4
						75					

TABLE 61.—BLOOD PICTURE IN CASE 2

TULAREMIA		TEMPERATURE	RED BLOOD CELLS	WHITE BLOOD CELLS	BASOPHILES	EOSINOPHILES	MYELOCYTES	JUVENILES	STABS	SEGMENTED	LYMPHOCYTES	MONOCYTES
December 2	-----	100.8	4,630,000	15,325	2	0	0	2	33	23	38	2
							58					
December 5	-----	99.6		12,050	2	2	0	2	25	24	42	3
							51					
December 7	-----	99.2		11,825	1	1	0	3	21	34	33	7
							58					
December 8. Agglutination Posi- tive 1:320	-----	99.4		14,500	1	1	0	2	22	32	38	4
							56					

Bubonic Plague

There is a neutrophilic leukocytosis, around 40,000. Platelets are increased. There is a very marked left shift.

Leprosy

The predominant feature of the blood in leprosy is an anemia. Leukocytes are not increased, eosinophilia is not common.

Erysipelas

There is a hyperleukocytosis with a marked regenerative shift to the left. Total neutrophiles may attain a figure of 90.

Encephalitis Lethargica

This is characterized by a slight increase of leukocytes with a moderate degree of anemia. There is a slight left shift. Some have reported a leukopenia and also an eosinophilia during the convalescent period. The type of encephalitis that occurred in St. Louis in 1933 showed a slight leukocytosis and a left shift.

Coccidioidal Granuloma

There is a moderate leukocytosis, usually around 15,000. Eosinophiles are sometimes above normal. Riesman and Ahlfeldt¹ reported a case with 22,000 white cells and 12 per cent eosinophiles. There is a secondary anemia.

Blood Picture in Appendicitis*

The data following represent a composite study of 97 consecutive operative cases of appendicitis. This analysis was made during our service at the St. Louis County Hospital. In all cases a hemogram was made, the clinical history studied, the operative findings recorded, and the gross and microscopic pathology of the appendix thoroughly analyzed. The purpose of the study was to correlate, if possible, the entire picture—clinical, histologic, and pathologic—and to attempt to draw conclusions on what one might expect from the blood picture as to the type of appendix to be found at operation.

For purposes of study and analysis, we made an arbitrary division of these clinical pathologic entities into five groups, as follows:

Group I. Thirty-one in number, which we decided to name *acute* appendicitis.

Group II. Twenty-seven cases in all, which we called *ruptured* or *necrotic* appendices.

Group III. Thirty-one cases in all, which we called *chronic* appendicitis with a flare-up or an acute exacerbation leading to operation.

Group IV. Five cases in all, characterized by *no definite pathology* in the coats of the appendix, aside from pressure effect from fecal impaction.

Group V. Three cases in all, which we are constrained to say exhibited no acute pathology, either gross or microscopic, which could be classified appendix inflammation.

We have selected a typical case from each one of the five groups for purposes of description.

Case No. 35-588, Group I.—History: Entered with pain in the right side, nausea but no vomiting. **Examination:** Tenderness in both lower quadrants, most pronounced on right side, with muscle guard and tenderness. **Hemogram:** 14,500 white count; 11 stabs, 58 segments, 30 lymphocytes, 1 monocyte. **Operation:** Day of entrance. Appendix found injected, some old adhesions to right tube. **Gross Examination:** Appendix about 6 cm. long with thickened wall; injected. **Microscopic Examination:** Mucosa markedly deep and friable in section; lymphoid elements in submucosa very abundant; tremendous invasion of lymphocytes and neutrophils into middle coat; small blood vessels filled with organized clots. **Microscopic Diagnosis:** An acute appendix inflammation.

*This is an abstract of a monograph by the author, read before the Pan American Medical Congress, General Medical Section in Havana, Cuba, January, 1938.

¹Riesman, D., and Ahlfeldt, F. E.: *Am. J. M. Sc.* 174: 151, 1927.

Case No. 35-598, Group II.—History: Entered hospital with pain in the right lower quadrant and in epigastrium. Vomited everything taken by mouth. *Examination:* Reveals a very sick child of 10, undernourished, abdomen greatly distended throughout, particularly over the right lower side. *Hemogram:* 17,800 white cells; no eosinophiles; 45 stabs; 35 segments; 15 lymphocytes; 5 monocytes. *Operation:* Same day, showed peritoneum filled with pus and fecal smelling fluid. Small fecalite found with perforation present at midpoint. *Gross Examination:* Appendix about 6 cm. long, tip very distended and discolored. *Diagnosis:* Ruptured appendix and peritonitis. *Microscopic Examination:* Very thick walled appendix with a very scant layer of mucosa. Intense infiltration of lymphocytes throughout all coats. The tissue appears necrotic as noted from the poor staining qualities of the entire area. *Microscopic Diagnosis:* Ruptured necrotic appendix.

Case No. 35-611, Group III.—History: About 2 weeks before entrance, patient noticed definite pains in right lower quadrant at irregular intervals. Nauseated but not vomiting. *Examination* at hospital showed no palpable masses, slight rigidity of abdominal muscles, some rebound tenderness. Center of pain in right lower quadrant in a line along McBurney's point. General impression was of chronic appendicitis. *Hemogram:* White cells 16,950; 14 stabs; 58 segments; 25 lymphocytes; 3 monocytes. *Operation:* Day following admission. Appendix found to be imbedded posteriorly and retroperitoneally; by blunt dissection and cutting of adhesion the appendix was finally brought into view. *Diagnosis:* Chronic appendicitis. *Gross Examination:* A long thin appendix 9½ cm. long. *Microscopic Examination:* A small appendix in width, very narrow. Mucosa layer very thin. Penetration of "wandering" cells and erythrocytes. Peritoneal coat distinctly thickened and elevated showing a chronic organization. Blood vessels engorged in the peritoneal coat. *Microscopic Diagnosis:* Process is essentially a chronic change in outer coat and slight change in the middle layer.

Case No. 36-085, Group IV.—History: Patient has been sick for 24 hours with epigastric pain, later localized in right lower quadrant, followed by nausea and vomiting. Vomited all night and 3 or 5 times on day of operation. *Examination:* Showed right muscle guard; marked tenderness over McBurney's point and Meltzer sign positive; rebound tenderness. *Diagnosis:* Acute appendicitis. *Hemogram:* White count 10,250; 15 stabs; 67 segments; 18 lymphocytes; no monocytes. *Operation:* Appendix found to be closely bound down by a membrane which was dissected free. *Diagnosis:* Acute appendicitis. *Gross Examination:* Appendix 7 cm. long; thick walled; filled with fecaliths. *Microscopic Examination:* Fecal impaction in the appendix that showed very little change in the wall beyond some slight penetration of lymphocytes and neutrophils into the middle coat. Outer coat showed very little change.

Case No. 35-691, Group V.—History: Several months previous had history of sharp lancinating pain in the right lower quadrant prior to onset of menstruation. This patient became slightly nauseated but did not vomit. Pain remained rather constant until termination of menses. This experience was repeated in the following two months' menstrual periods. Patient became slightly more nauseated with each attack and of late has had backache and morning nausea. On no occasion has patient vomited with these attacks. *Examination:* No masses. Tenderness in both lower quadrants, especially right and over McBurney's point. No muscle guard or rigidity, no rebound pain. Right inguinal quadrant tender. Left inguinal quadrant negative. Impression: Chronic appendicitis, chronic salpingitis, ovarian cyst. *Hemogram:* 8,000 white blood cells; 1 eosinophile; 3 stabs; 62 segments; 30 lymphocytes; 4 monocytes. *Operation:* Showed some adhesions between appendix and the right adnexa. Right tubal wall thickened. *Gross Examination:* Appendix 7 cm. long. Thick walled. *Microscopic Examination:* Very thin wall; no changes in the submucosa lymphoid elements; narrow mucosa with few migrating or wandering cells in any of the layers. Muscular coat very thin. Peritoneal coat showed some very slight chronic changes in the shape of elevation with new elements of fibrous connective tissue beneath the outer layer. In general, this might be termed an atrophic appendix.

In order to make the findings more graphic, we confined the hemograms in this work to the following data: total white count, and number of stabs and segments comprising the total neutrophilia.

A study of the total count, differential count, and the febrile reactions, together with a knowledge of the clinical history, operative conditions, and pathology, permits one to draw certain very definite conclusions regarding inflammation of the appendix, diagnosis, and operability. It is evident that in all cases of disturbances of the appendix there is likely to be an increase in the white cells, together with a left shift. This left shift is likely to proceed farther to the left in cases of ruptured, gangrenous, and necrotic appendices than in any other type. In other words, the only differentiation which is possible from the blood picture between cases of the first group and those of the second group is possibly a more severe shift to the left. Certainly, the amount of the total count in the two types is not likely to be different. It is a fact, however, that in cases of ruptured appendices the total count begins to go down with an increase in the severity of the pathologic conditions of this variety; in other words, with probably decrease in resisting power of the organism.

Therefore the hemogram is of some assistance in elaborating the diagnosis of appendicitis; *one is in no way able to predict from the hemogram alone the exact degree of pathology which will be found at operation or after microscopic study.* Some clinical pathologists and surgeons attempt to draw conclusions of too refined a character from the hemogram in the presence of a disturbance in the right lower quadrant. We wish to emphasize that this study, together with other experiences, would cause us to hesitate to draw any conclusion other than the presence of inflammation of the appendix from any given hemogram. We do, however, acknowledge that a falling total count and an increased left shift is a prime indicator of a probable rupture with a severe peritonitis and possibly a fatal outcome. It can be noted that, first, there is an irregular fever in all types of appendix inflammation; second, there is a difference in the degree of symptomatology; third, there is very little difference in the height of the white count and in the left shift in most cases whether they belong to the acute group, so-called, or ruptured appendicitis, or chronic appendicitis with acute exacerbation. The distinction, if any, may possibly be a more pronounced left shift, but this is not a sufficient basis upon which to make an absolute differentiation. The blood picture in the chronic cases with intermittent flare-ups shows leukocytosis, together with a left shift, although this leukocytosis in a general way may not be as high as that commonly found in Group I or Group II. The obvious conclusion to be drawn from this investigation is that the surgeon must make up his mind as to the diagnosis from the clinical symptoms, the range of fever, and the blood picture; he must not attempt to prognosticate as to just what pathology he is likely to find from the blood picture alone. Attention, too, must be called to the fact that in the case of fecal impaction without much pathology in the appendix wall, the symptoms were mild but decisive, the blood count was increased in a total way, and there was a mild but definite left shift. In all probability, cases of this nature, if left alone,

would have resulted in an extension of the pathology to the wall of the appendix, possibly leading to rupture and peritonitis had they not been operated upon. A fecal impaction in the appendix must be regarded as a foreign body, which is a source of definite trouble and which may be a potential source of danger to the patient, a cause for removal.

Whether to operate or not and when to operate seem to be the much discussed points in surgical circles regarding inflammation of the appendix. If the pathologist is asked for his opinion on this question, he must unhesitatingly answer, from a study of these cases, that once a diagnosis is made, an operation should follow. There seems to be no criterion by which one may conclude that in a given acute case subsidence of the symptoms is likely to occur and an interval operation may be indicated. From the viewpoint of pathology, therefore, one must urge operation once a diagnosis has been established.

The three cases in women, in which the diagnosis was not substantiated by operation or pathology, were all cases of right-sided tubal difficulties. Possibly, in these cases, the use of the blood sedimentation test would have assisted in making a laboratory differentiation between inflammation of the appendix and right tubal difficulty. At this point, the value of blood sedimentation as one of the nonspecific methods of laboratory diagnosis must be emphasized.

Conclusions.—The use of the Schilling hemogram in appendix cases offers many advantages. It enables us to draw a very fine picture of the reaction of the organism to infection in the shape of a response of the bone marrow. The first effect of infection on the bone marrow is paralysis of the function, resulting in a temporary leukopenia. Continuance of the infection results in a stimulation with an outpouring of immature forms of neutrophiles into the circulation. Coincident with this is the disappearance of eosinophiles and a relative lowering of the lymphocyte count. Usually, too, there is a decrease in the number of monocytes. With improvement, eosinophiles reappear, monocytes attain a high level, and lymphocytes increase, together with a recession of the neutrophiles from the left to the right of the hemogram. Definite conclusions may be drawn by the laboratory worker concerning the reaction of the organism to the infection. All attempts, however, definitely to dictate the entire diagnosis and prognosis from the hematologic picture are to be discouraged. There should be an objective interpretation from the laboratory standpoint which is manifestly suggestive concerning the presence of infection and the degree of immune reaction. With no clinical data before him, this is as far as the hematologist should go. With the clinical data added to the hematologic picture, very definite conclusions may be drawn as to the reaction of the organism, the possibility of a serious infection, and the probability of recovery. The clinical facts and the hemogram enable us to form a prognostic picture. It is to be emphasized that the hematologist and the clinician must work hand in hand on the interpretation of the Schilling blood picture. If this is done, untold advantage accrues. If it is not done, there may be embarrassing moments when the hematologist presumes too much and draws conclusions from the blood picture alone. The conclusion is obvious from a study of the analysis of these cases that in appendicitis there are definitely a left shift and a leukocytosis.

The severity of this shift does not enable one to differentiate between an acute case and one that represents the flare-up of the so-called chronic or subacute case. The hematologic facts at once indicate that there is a definite pathology in the appendix. There is no method by which one may predict a subsidence of the infection, and the action of the so-called interval operation. In other words, with the left shift, the leukocytosis, and the clinical symptoms, diagnosis of the appendicitis ought to be determined immediately and the correct treatment ought to be instituted, namely, operative procedure. The ruptured cases, of course, show decreasing total counts and increasing left shifts. This has been the belief for many years. It has been amply confirmed in these cases.

The following hemograms represent typical findings in appendicitis (not part of the series of 97 just recorded).

Acute Appendicitis (Fatal—too late to operate).

White Count 27,800. Red Count 3,280,000. Hb. 60%.									
Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.	
0	0	4½	19	51½		12	12½	½	
Polychromasia ±.									

Acute Appendicitis.

White Count 22,500. Red Count 4,490,000. Hb. 85%.									
Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.	
0	0	0	1	26		60	13	0	

Acute Appendicitis.

White Count 13,800. Red Count 4,604,000. Hb. 80%.									
Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.	
0	0	1	10	59		20	10	0	

Acute Appendicitis.

White Count 22,057.									
Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.	
0	0	0	0	18		66	14	2	

Amebiasis

In amebic dysentery there is a secondary anemia and, in severe chronic processes, the erythrocyte count may drop to as low as 2,000,000, with a still greater reduction in hemoglobin. There is a hyperleukocytosis and a marked left shift. Eosinophilia is insisted upon by some and denied by others. It is a general belief of hematologists that the presence of eosinophilia indicates infestation with intestinal worms as a complication.

When amebic abscess of the liver supervenes, there is no special increase in leukocytes.

		Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
First day	10,850	0	12	0	0	5		48	33	2
Second day	10,450	0	12	0	0	5		55	23	5

Allergic States

The majority of hematologists regard eosinophilia, together with a leukopenia, as a very marked sign of allergy (see Leukopenic index, page 1357).

Scarlet Fever (Scarlatina)

The so-called "gay" blood picture with increase of eosinophiles is characteristic of scarlet fever. Most cases show a marked neutrophilic leukocytosis. Values of 20,000 per cu.mm. in severe cases are often found. In milder cases, the count is around 10,000. The white cells are increased coincident with the exanthematous eruption. With decline in fever, the white cells gradually decline. In this disease, we find a persistent leukocytosis, sometimes for a long time after the decline of the fever. The count is usually back to normal at the time desquamation is finished.

Increase in the eosinophiles begins shortly after the onset. In the beginning of the disease, the increase is confined to the neutrophiles. The neutrophiles show changes in the nuclear structure with degeneration in the cytoplasm, with the formation of vacuoles, and basophilic clumping, the so-called Doehle inclusion bodies. (For further details see remarks on toxic granules on pages 648 and 650.) They are plump and usually heavily stained granules. Cocci are sometimes seen within the cells. Monocytes are numerous. There is a relative and absolute increase.

Roth claims that there is a sudden increase of neutrophiles in scarlet fever, and then a gradual rise to higher levels, with a postinfectious lymphocytosis, without increase of monocytes. Plasma cells are sometimes found, according to Ciuca,* and may be as high as 17 per cent.

The most interesting feature of the scarlatinal blood picture is the eosinophilia. In the first two days, the eosinophile count is low, increasing after the second or third day of exanthematous eruption. The following hemogram represents a case on the second day of the eruption:

Bas.	Eos.	Myel.	Juv.	Stabs	Seg.	Lymph.	Monos.
0	$\frac{1}{2}$	0	1	15	$57\frac{1}{2}$	$18\frac{1}{2}$	7

Plasma cells: $\frac{1}{2}\%$. 1 monocyte with lobulated nucleus.

The following hemograms are quoted from Naegeli:

Second Day. Total 16,680.

Bas.	Eos.	Neutrophiles	Lymph.	Mono.	Plasma Cells
0.1	2.3	83.9	5.2	8.1	0.4

Fourth Day. Total 15,520.

Bas.	Eos.	Neutrophiles	Lymph.	Mono.	Plasma Cells
0.4	5.3	68.0	17	8.5	0.9

According to Tuerk¹ scarlatina may show as many as 14 per cent eosinophiles. Naegeli found as many as 22 per cent. He has one record of 25 per cent in a leukocyte count of 10,000.

The maximum period for eosinophiles usually occurs at the end of the first week. In very severe and in lethal cases, the eosinophilia is absent, according to Ambrus,² Georgescu,³ Markovitch,⁴ and Ketschetkoff.⁵ Naegeli reports that on the third day of a case of severe septic scarlatina he found

*Ciuca: *Compt. r. Soc. de biol. Paris* 98: 1627, 1928.

¹Tuerk: *Monatschr. f. Kinderh.* 15: 1918.

²Ambrus: *Jahrb. f. Kinderh.* 101: 68, 1924.

³Georgescu: *Inaug. Dissert. Bukarest*, 1911.

⁴Markovitch: *Presse méd.*, 205, 1925.

⁵Ketschetkoff: *Zentralbl. path. Anat.*, 1892, No. 11.

only 0.5 per cent eosinophiles; on the fourth day the case ended fatally. Heubner and Roth⁶ also reported cases of septic scarlatina without eosinophilia.

The question has been raised—does the cause of the eosinophilia lie in the reaction of the skin in scarlatina? It is claimed that those cases which fail to show an eruption do not show eosinophilia. Schemensky⁷ observed one case of scarlet fever without an eruption and insisted that eosinophilia is due to the presence of some eosinophile-chemotactic substance in the skin which is increased in the period of exanthem. Basophilic cells also have been found increased in this disease sometimes as high as 4 per cent. A few myelocytes have been found, 3 per cent in a total count of 16,000 having been recorded.

There is always a severe secondary anemia in scarlatina. Occasionally nucleated reds have been observed.

The following hemograms were made from cases in the St. Louis Isolation Hospital, through the courtesy of the Superintendent, Dr. John Eschenbrenner:

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	4	0	6	30.5		35	12.5	9.5

Lymphoblasts (?) 0.5 per cent, 1 normoblast, red cells show basophilic punctation and polychromasia 2+. 2% plasma cells.

Second day of eruption:

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	3.5	0	0.5	10		38.5	40	7

0.5% plasma cells, basophilic punctation 2+.

From case four days after eruption began:

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	4	0.5	1.5	17		42	19.5	11.5

4% plasma cells.

Hemogram of mixed infection scarlatina and measles, four days after onset:

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	3.5	0	1.5	24.5		42	20.5	7.5

Plasma cells 0.5%, marked polychromasia.

Diphtheria and Scarlatina fifth day:

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	3	0	0	12		62	12.5	9.5

1% plasma cells.

The following hemograms are also illustrative of scarlet fever:

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	6	0	1	17½		47½	23	4½

Plasma cells ½%.

Ninth day.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	3	1	3½	17		35	28½	10½

Plasma cells 1½%.

⁶Heubner and Roth: Med. Klin. 1910.

⁷Schemensky: Zentralbl. f. inn. Med. No. 26, 1918.

Scarlet fever and measles.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	2	0	$\frac{1}{2}$	6		47	34	10

Plasma cells $\frac{1}{2}\%$.

Scarlet fever and diphtheria.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	$2\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	$24\frac{1}{2}$		49	$15\frac{1}{2}$	6

Plasma cells $1\frac{1}{2}\%$.

Measles

In measles there are no characteristic changes in the hemoglobin content value or the count of the red cells. At the beginning of the disease there is a neutrophilic leukocytosis followed rapidly by neutrophilic leukopenia. The leukocytes appear during incubation, and the leukopenia is seen at the time of eruption. There is almost a continuous leukopenia, the lowest count recorded being 2,400, during the height of the disease. Usbeck found a leukopenia in the beginning inconstant. Hecker found an incubation period at the time of the appearance of the Koplik spots, a leukopenia as well as a relative and absolute lymphocytopenia with left shift and a decrease in eosinophiles. In the second half of the period of incubation, there is an increase in lymphocytes and a decrease in neutrophiles.

In the exanthematous period there is a leukopenia, absolute and relative decrease or absence of eosinophiles. This typical blood picture was found twelve times in forty-one cases examined. In recrudescence, there is a leukocytosis and a relative lymphocytosis and eosinophilia. There is not a left shift.

With complications, such as pneumonia, otitis, etc., we may find a leukocytosis, neutrophilia, and a left shift. The blood platelets at the beginning of the exanthematous period are decreased to 100,000.

During the eruption there is a marked left shift with predominance of young forms. There are plasma cells and the usual "gay" blood picture of Schilling. The following hemograms are typical of this disease:

W. H.

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
0	$10\frac{1}{2}$	0	0	5		20	52	7

Polychromasia and basophilic punctation. Plasma cells, $5\frac{1}{2}\%$.

G. H.

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
0	2	0	1	10		24	$51\frac{1}{2}$	7

Plasma cells, $4\frac{1}{2}\%$.

German Measles

The hemoglobin value and the red cell count are not characteristic. Leukocyte count is decreased or normal. The minimum count of the leukocytes is found on the third day after the eruption. The number of neutrophiles is absolutely or relatively decreased, while the lymphocytes and monocytes are increased. The eosinophiles and basophiles are unchanged. There is a characteristic "gay" blood picture, with numerous plasma cells.

Psittacosis

In psittacosis there is an absence of leukocytosis at the beginning of the infection. Leukopenia is present in a small number of cases. When the disease is at its height, there is a shift to the right of the neutrophils, accompanied by a distinct fall in lymphocytes. As the patient improves, normal distribution of the blood cells follows. There is a distinct acceleration of sedimentation time at the beginning of infection.

Varicella

There is no change in the hemoglobin and the red cells in this disease. Arneth found during the eruptive period of the disease in a 2-year-old boy a white count of 6,000, and with recovery, a restitution to normal figures. There is a left shift at the beginning, but this rapidly disappears. Nobecourt and Merklen reported fifteen cases, ages 1 to 5, without any characteristic changes.

In the suppurative stage, there is a characteristic left shift which decreases with the decline of suppuration. So far as the blood platelets are concerned, they decrease with the beginning of fever and increase with the decline of fever.

Variola

The blood picture of variola is characteristic and constitutes a very important link in the diagnostic chain. We find early in the disease a marked regenerative shift as follows (on the fourth day):

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
0	5	1	6.5	8		25	31	23.5

together with a marked hyperleukocytosis. We are also likely to find many plasma cells, 11 per cent plasma cells having been recorded. According to Schilling, there is a marked left shift and an increase in monocytes—not only the normal monocytes are increased but there are abnormal large juvenile forms of monocytes. Fischer has described myelocytes and large lymphocytic irritation forms in this disease.

Diphtheria

There is a characteristic leukocytosis in diphtheria. With the injection of diphtheria antitoxin, there is a rapid decline in the number of leukocytes. Schlessinger¹ investigated leukocytosis in experimental diphtheria in animals. After injection of diphtheria bacilli, he noted leukopenia together with a decrease in lymphocytes, and then came an increase in leukocytes. Heim² claims that leukocytosis runs parallel with the intensity of the infection. Others note an absolute and relative increase in neutrophils, together with a decline in lymphocytes in the beginning of the disease. Our own hemograms follow:

Eight days' duration—I. R.—

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
0	1	0	0	16		52	23	8

¹Schlessinger: Die Leukozytose bei Diphtherie, Arch. f. Kinderh. 19: 378, 1896.

²Heim: Arch. des mal. des enf. 48: 24, 1901.

Twenty-two days' duration—W. R.—

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
0	0	0	4½	15		56½	18½	5

Plasma cells ½%—marked polychromasia.

Pertussis

In pertussis, there is a leukocytosis, sometimes as high as 40,000 or higher. The younger the child, the more outstanding the leukocytosis. This leukocytosis is practically a lymphocytosis. In twenty-four cases in the catarrhal stage, there was an average of 20,000 white cells, of which 32 per cent were neutrophils and 66 per cent were lymphocytes. The highest leukocytic and lymphocytic figures are found during the paroxysm of the disease. Complications by streptococcus, pneumonia, or staphylococcus do not seem to influence the count. Thiemann found in a typical pertussis case, 42,000 leukocytes with 76 per cent lymphocytes and 19 per cent neutrophils.

There is a left shift of the neutrophils in either the catarrhal or paroxysmal stages. Count of the eosinophiles is not changed. Monocytes are normal at first but become slightly elevated later. In complications, there is likely to be a left shift in the neutrophils without any change in the lymphocytes.

Parotitis Epidemica

Lehndorf¹ reported the blood pictures of fourteen cases of mumps in adults. Leukocytes were normal. Lymphocytes were increased 30 to 65 per cent. Two per cent plasma cells were found. Neutrophils were decreased; likewise eosinophiles.

Wiese,² in 115 cases of mumps from the ages of 5 to 15, found the following (no changes in the hemoglobin and red cell count) :

Day of Disease	Bas. %	Eos. %	Myel. %	Juv. %	Stabs %	Segs. %	(Neutr.) %	Lymphs. %	Monos. %	Neutr.	Lymphs.	Total Leukocytes
1	—	—	—	—	—	—	—	—	—	—	—	—
2	1	5	—	2	9	21	32	47	15	1,760	2,585	5,500
3	2	5	—	1	6	14	21	59	13	1,144	3,216	5,450
4	1	3	—	—	1	11	12	68	10	0,972	5,508	8,100
5	1	12	—	1	4	17	22	57	8	1,771	4,589	8,050
6	—	6	—	1	1	27	29	55	10	2,088	3,960	7,200
7	1	7	—	2	2	36	40	48	4	4,080	4,896	10,200
10	—	—	—	—	—	—	—	—	—	—	—	11,600
11	1	7	—	2	2	40	45	44	3	4,896	6,820	13,000

There is a marked left shift and lymphocytosis together with a leukopenia in the beginning of the disease.

Miscellaneous Cases

Staphylococcic Septicemia

(1)
(Fifteenth Day.) White count 14,950. Red Count 4,600,000. Hb. 90%

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	0	0	1	23		65	8	3

¹Lehndorf: Wien. klin. Wchnschr. 31: 560, 1918.
²Wiese: Arch. f. Kinderh. 80: 253, 1927.

(Twenty-ninth Day.) White Count 18,550.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	0	0	0	25		56	17	2

Patient improved—left hospital.

(2)

White Count 34,950. Red Count 4,950,000. Hb. 95%. CI 0.9.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	0	0	12	31		46	8	3

Ectopic Pregnancy

(1)

White Count 18,100. Red Count 3,840,000. Hb. 40%.

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymph.	Monos.
0	1	0	0	9		76	14	0

(2)

White Count 12,500. Red Count 4,640,000. Hb. 75%.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	0	0	0	6		80	13	1

Ovarian Abscess

White Count 9,900. Red Count 4,540,000. Hb. 85%.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	2	0	0	12		59	25	2

Abscess of Tube and Ovary (Old)

White Count 8,450. Red Count 4,220,000. Hb. 85%.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	5	0	0	14		31	45	5

Polychromasia \pm .**Salpingitis**

White Count 14,700. Red Count 4,610,000. Hb. 90%.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	2	0	0	9		47	39	3

Cholecystitis (Chronic)

White Count 8,700. Red Count 4,670,000. Hb. 90%.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	2	0	0	5		67	25	1

4/19—White Count 13,800. Red Count 4,870,000. Hb. 90%.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	1	0	0	1		58	36	4

Progressively worse.

5/13—White Count 11,750.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	2	0	0	5		72	17	4

Cholecystitis (Acute)

White Count 13,050. Red Count 4,320,000. Hb. 85%.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	0	0	0	6		73	18	3

White Count 18,350. Red Count 4,800,000.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	1	0	2½	13½		64½	14½	4

White Count 28,800. Red Count 5,070,000.

Bas.	Eos.	Myel.	Juv.	Stabs		Seg.	Lymph.	Monos.
0	0	0	2	13		72	11	2

Septic Abortion

The following two cases show a very interesting condition. They are both cases of septic abortion; one terminated favorably and the other fatally. The one that terminated favorably had a very marked hyperleukocytosis and a marked left shift. The other, which terminated fatally, had leukopenia and a very marked left shift. The latter is always to be regarded as a very unfavorable condition; namely, a low white count and a marked left shift. This is found in severe infections preceding death and in cases of ruptured appendix with widespread peritonitis, which have a fatal ending.

Mrs. W.—

10/30. White Count 70,400.

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
0	0	1	13	43		42	1	0

10/31. White Count 45,600.

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
0	0	0	11	46		36	5	2

11/4. White Count 31,850.

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
0	0	0	6	37		43	11	3

11/9. White Count 17,300.

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
0	2	0	3	29		42	22	2

Patient improved.

Mrs. S.—

White Count 6,250.

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
0	0	2	17	43		30	7	1

Patient expired.

Neutrophilia With Right Shift—Polyarthrititis Acuta

There is one condition in which one is likely to find a neutrophilia with right shift—polyarthrititis acuta. The following case reports from our service at the St. Louis County Hospital offer typical pictures of this condition:

W. D., aged 33, entered hospital with history of chills, fever, cough, and general malaise, diagnosed as influenza six weeks ago. Later had pain in lower back and left thigh on lying down, and left thigh began to flex on the abdomen. X-ray showed osteoarthritis involvement in the lower lumbar vertebrae, pelvis, and hip. Pelvis was markedly distorted. Diagnosed as hypertrophic osteoarthritis with pelvic distortion. Hemograms are submitted as follows:

DATE	TEMP.	W.B.C.	R.B.C.	H.B.	CI.	B.	E.	M.	J.	STAB	SEG.	L.	M.
1	103.2	26,750	4,330,000	85	0.9					3	85	10	2
2	101.4	27,150					1			5	80	11	3
3	100.8	21,550					3			5	80	10	2
4	99.2	30,200								9	82	8	1
	(Patient's leg was stretched)												
5	98.4	21,700							1	9	80	6	4
6	98.2	20,050					1			9	81	6	3
7	98.6	16,250					4			6	79	9	2
8	99.0	15,600					3			6	75	14	2
9	100.0	13,000					4			7	72	13	4
10	99.6	9,650					4			6	67	17	6
11	100.4	15,850					1		1	9	66	17	6
12	100.6	14,250				1	1			5	71	20	2

C. D., aged 54, entered St. Louis County Hospital with history of painful swollen knee for one year; both feet swollen and painful for past six months. Heart enlarged, systolic murmur. X-ray report as follows: the right knee shows hypertrophic osteoarthritis with calcium deposits within the soft tissue and at the borders of the lower head of the femur. The tibial spines are accentuated. There is evidence of arteriosclerosis. The synovial structures are thickened about the right knee. The left knee shows similar pathology but to a lesser degree. The right ankle and foot show atrophy of bone with some erosion of the articular structures. Arteriosclerosis is also present. Diagnosis: Arthritic involvement of both knees and ankles, more marked on the right side.

Hemograms in this case showing leukocytosis, neutrophilia, and right shift are as follows:

DATE	TEMP.	W.B.C.	R.B.C.	HB.	CL.	B.	E.	M.	J.	STAB	SEG.	L.	M.
Dec. 2/33	100.8	14,450	4,871,000	95	0.9		2			7	65	24	2
Jan. 13/34		19,350	5,010,000	95	0.9		5			3	58	29	5

MISCELLANEOUS DISEASES OF THE BLOOD AND BLOOD-FORMING ORGANS

TABLE 62.—MISCELLANEOUS DISEASES OF THE BLOOD AND BLOOD-FORMING ORGANS*

TERM TO BE USED	TERMS TO BE AVOIDED
<i>Agranulocytosis</i> , due to Unknown cause Drugs Granulocytic hypoplasia, from Unknown cause Drugs Radiation <i>Infectious mononucleosis</i> <i>Infectious lymphocytosis histiocytosis</i> , due to Unknown cause Lipidosis, of Unknown type Kerasin type Cholesterol type Phosphatid type Myeloid metaplasia of spleen, due to Unknown cause Osteopetrosis Myelofibrosis	Malignant granulocytopenia, agranulocytic angina Glandular fever, benign lymphadenosis, monocytic angina, acute benign lymphoblastosis, lymphocytic angina, infective mononucleosis Gaucher's disease, metaplastic reticular and histiocytic cerebrosiderosis Hand-Schüller-Christian disease, xanthomatosis Niemann-Pick's disease, metaplastic reticular and histiocytic sphingomyelinosis Agnogenic myeloid metaplasia

*Reproduced from the Am. J. Clin. Path. 20: 574-579, 1950, by courtesy of the Editor and of the Williams and Wilkins Company, Baltimore.

Agranulocytosis

This disease has been called by various names. "Agranulocytosis" was the name given to the disease by Schultz,¹ who first described it. Schilling called the disease malignant neutropenia, which is a better designation than Schultz' term, which means literally a condition where there are no granulations in the neutrophilic cell. As a matter of fact, in this disease there is a marked diminution in the number of neutrophilic cells. It has also been called

¹Schultz, W.: Deutsche med. Wchnschr. 48: 1495, 1922.

angina agranulocytica, mucositis necroticans agranulocytica, agranulocytosis septicaemica simplex, stomatitis gangrenosis myelophthisica, malignant leukopenia, hypogranulocytosis, and sepsis with granulocytopenia. This condition was said by Türk to be a disappearance of the neutrophilic system.

Etiology.—It has been claimed to be largely due either to the influence of drugs or to a bacteriologic causative factor. It has been known to follow arsenical and bismuth injections. Madison and Squire² reported thirteen cases in which they thought this condition was due to the use of benzene chain derivatives. Watkins³ discussed the role played by barbiturates and amidopyrine in the causation of neutropenic states. Hardwick and Randall⁴ report a number of cases of parturition in which large doses of phenobarbital-sodium were given without once noting any development of neutropenia, although some have claimed that these drugs will cause malignant neutropenia. These writers did show, however, that a high degree of leukocytosis appears at the fifth hour post partum, which has, of course, nothing to do with the explanation of the etiology of agranulocytosis (see page 788).

Von Bonsdorff⁵ reported three cases with malignant neutropenia. In two cases it was virtually certain that medication with amidopyrine was the cause, and it was probably the cause in the third case. In some persons a few small doses of amidopyrine produce granulocytopenia, while in other cases it appears suddenly some time after large doses of the substance have been administered, without causing disturbance at the time of administration. This shows that therapeutic doses of amidopyrine in sensitive persons may have a detrimental effect on the general condition and on the hematopoietic organs. The action on the granulocytopoietic apparatus is stimulating as well as inhibiting. The total number of granulocytes decreases, but the immature and young forms increase. In some instances leukocytosis may follow. Amidopyrine influences the organs that produce the monocytes and lymphocytes as well as the erythrocytes. The changes disappear spontaneously when medication is discontinued. However, it may be assumed that the continuous use of amidopyrine may eventually cause grave clinical symptoms. Animal experiments proved likewise that the administration of amidopyrine or of related substances may eventually impair the hematopoietic organs and lead to leukocytosis, leukopenia, or anemia.

Kracke⁶ pointed out that coal tar products may be responsible for this disease. In one case he claimed that acetophenetidin was probably responsible for this disease. There have been attempts made to differentiate between the etiology of the acute cases and the so-called chronic cases. Roberts and Kracke⁷ were impressed by the increasing number of acute fulminant cases and the large number of so-called chronic cases. They claimed that the acute cases represent a very small percentage of the total number of individuals whose blood count is depressed to some degree. Kracke and Parker⁸ de-

²Madison, F. W., and Squire, T. L.: *J. A. M. A.* **102**: 755, 1934.

³Watkins, C. H.: *Proc. Staff Meet., Mayo Clinic* **8**: 713, 1933.

⁴Hardwick, R. S., and Randall, L. M.: *J. A. M. A.* **102**: 1558, 1934.

⁵Von Bonsdorff: *Klin. Wchnschr. Berlin* **14**: 449, 1935.

⁶Kracke, R. R.: *Am. J. Clin. Path.* **1**: 385, 1931.

⁷Roberts, S. R., and Kracke, R. R.: *Ann. Int. Med.* **5**: 4, 1931.

⁸Kracke, R. R., and Parker, F. P.: *J. Lab. & Clin. Med.* **19**: 779, 1934.

scribed the effects of drugs containing the benzene ring on agranulocytosis. They state that a certain percentage of the cases are due to arsphenamine, a certain percentage to gold salts and perhaps other chemicals, leaving a large group of the idiopathic type with an unknown etiology. Since the disease is more prevalent among physicians and their relatives, nurses, and members of the allied professions than in any other group in the United States, it may be due to the ingestion of drugs which these professional persons are in the habit of using rather freely, that is, drugs of the benzamin group. In eleven cases investigated by Roberts and Kracke, various drugs were taken, such as amidopyrine and Empirin compound, phenacetine, acetanilid, amidopyrine, and Allonal. These cases did not include the chronic type. These investigators further studied the groups of people who are likely to use coal-tar drugs in a rather promiscuous manner, and they found in an investigation of nurses, students, physicians, etc., that 76 per cent ordinarily used a drug containing the benzamin group. It is their contention that in persons with a weakened, damaged, or idiosyncratic bone marrow, either acquired or congenital, the use of these drugs may produce the chronic form of malignant neutropenia.

It has been proved that drugs may affect the bone marrow in certain peculiarly sensitive individuals. For instance, purpura following quinine administration has been observed. Benzene administration may be followed by aplastic anemia. Krumbhaar noticed profound leukopenia and marked changes in the bone marrow from mustard gas poisoning. Dinitrophenol, a drug which was widely used for the treatment of obesity, has been followed by agranulocytosis. The amount of the drug and the period of time over which it is taken are unknown factors in relation to the development of this disease.

Infection is a factor that has been stoutly maintained by a number of observers. No specific organism has been shown to be consistently capable of producing agranulocytosis. Blood cultures in many cases have shown a variety of organisms. In some cases the culture is positive, in others, negative. *Streptococcus pyogenes*, *Escherichia coli*, streptothrix, pneumococcus, and others have been found in different cases.

Moeschlin and associates have shown the development of antibodies against white blood cells. Refer to section on Immunohematology for further information.

The disease begins usually with a sore throat of sudden onset. Occasionally, however, there is a history of long-continued sore throat. There is fever and prostration. It behooves the physician in all cases of sore throat to make a blood picture in order at least to eliminate the possibility of this disease. Examination of the blood will usually suffice to clinch the diagnosis, although often the hemoglobin and red count estimations are normal. Occasionally, there may be an unexplained severe anemia, but this is not common. The number of blood platelets may be normal or decreased. The white blood count is the most characteristic feature of the disease. There is a diminution of the total number, which may be down to 1,000 white cells. We have seen cases with counts as low as 500. The lower the total count, the fewer are the granular elements; so much so, that finally no granulocytes are seen at all

in the blood spread. The differential count shows a complete disappearance of neutrophilic leukocytes with relative lymphocytosis. Plasma cells and myeloblasts are occasionally found. The number of monocytes may be diminished. In fatal cases the number of white cells remains low and shows a progressive decline. Gradual increase in the percentage of neutrophiles and in the number of leukocytes is a favorable symptom. The nuclei of the neutrophilic segmented cells are blurred. The neutrophilic granules appear basophilic and coarse. These are the toxic granules first described by Cesaris-Demel. (See pages 648 and 650 for discussion of toxic granules.)

Blood Findings and Histories of Agranulocytosis Cases.—

Case 1.—A very interesting case of this disease in the author's service at the Christian Hospital is submitted through the courtesy of Dr. Melvin Staehle.

Admission complaints and findings: The patient, a 46-year-old woman, was admitted April 28, complaining of becoming acutely ill on April 24, with a chill, fever, severe sore throat, inability to swallow, headache, dizziness, and general malaise.

The appetite was poor, patient very constipated.

The abdomen was soft and distended. The liver and spleen were not enlarged. No tenderness or masses palpable. Heart sounds of fair quality; no murmurs.

Vesicular breathing; no definite dullness. A few moist râles, mostly at bases.

No renal tenderness, kidneys not palpable.

Reflexes physiologic.

Acute inflammation of the pharynx.

April 28, 1942.

Upon admission: Respiration 26. Temperature: 104°. Pulse 80.

Sulfathiazole was given.

April 29, 1942.

Sulfathiazole discontinued.

Pentnucleotide 10 c.c. 4 times daily ordered.

250 c.c. blood as transfusion. All blood taken. A severe reaction followed, with a chill, nausea. Lasted twenty minutes.

May 1, 1942.

Patient belonged to Group A₁. 250 c.c. blood, Group A₁, as transfusion; no reaction.

May 2, 1942.

250 c.c. blood, Group A₁, transfusion; no reaction.

May 3, 1942.

250 c.c. blood, Group A₁, transfusion. Discontinued, chill, headache.

May 5, 1942.

Pentnucleotide reduced to 2 times daily.

May 9, 1942.

Pentnucleotide discontinued, as the patient had been without fever for 4 days.

May 10, 1942.

Discharged from the hospital.

HEMATOLOGY OF THIS CASE

DATE	R.B.C.	W.B.C.	HGB.	C.I.	E.	B.	M.	J.	ST.	SG.	L.	MO.	PL.C.	IRR.C.
4/29/42	3,670,000	3,750	77% S.	1.0	0	1	0	0	0	0	96	3	0	0
4/30/42		1,400			0	1	0	0	0	0	97	2	0	0
5/ 1/42		1,250			0	0	0	0	4	24	66	5	0	1
5/ 2/42	3,590,000	2,000			0	2	0	3	7	4	64	20	0	0
5/ 3/42	4,900,000	3,600	93% S.	0.9	0	0	1	13	13	22	35	16	0	0
5/ 4/42		6,050			0	0	2	19	13	40	18	8	0	0
5/ 5/42		6,250			0	0	1	7	13	41	29	8	1	0
5/ 6/42		6,800			0	1	0	8	10	33	36	12	0	0
5/ 7/42		8,650			0	0	0	7	10	35	26	17	4	1
5/ 8/42		13,250			0	0	0	2	7	45	21	25	0	0
5/ 9/42		9,050			0	0	0	2	4	62	20	12	0	0
5/10/42	5,000,000	10,000	105% S.	1.0	0	1	0	3	3	51	32	9	1	0

Case 2.—The following case history is from our service at Christian Hospital, St. Louis, Mo., and is presented with the permission of the attending physician, Dr. H. A. Klein. Patient, Mrs. S. K., was admitted to the hospital on Dec. 9, 1942, at 2:30 P.M. Complaint, sore throat of three days' duration; chills, fever, and general malaise. Family history, irrelevant. Personal history, usual childhood diseases; no other infectious diseases; no serious operations or injuries; has been housewife all of married life; no history of exposure to chemicals. History of present illness: Patient began having a "cold" five days before entry into hospital. Three days before entrance her throat began to feel sore, soon becoming so much so that she could not swallow. She has had some generalized aching pains, especially of the shoulder and hip joint, chills, fever, and sweating. The patient was told five weeks before entering the hospital that she had high blood pressure and was given amidopyrine gr. ii ss dosage, which she took for two weeks. She has also been given medicine for her "liver." Patient has not taken any sulfonamide drugs or headache powders.

Examination of the patient revealed the following. General condition: Well-nourished 65-year-old female patient who looks acutely ill in fair condition. Tongue coated; abdomen soft and distended; liver slightly enlarged; spleen not palpable; no tenderness or masses palpable; heart slightly enlarged especially on the left; no murmurs, sounds of fair quality, aortic sounds accentuated; pulse fast, regular, equal, well filled; blood pressure 150/120; vesicular breathing; no dullness, few moist râles; bases move; no renal tenderness; kidneys not palpable; nervous system: reflexes, questionable Babinski at right (voluntary?); pupils round, equal, react to light; acutely inflamed throat, necrotic spaces; extremities, no edema or injuries. Impression: agranulocytic angina.

Records of temperature, pulse, respiration, urinalysis, and blood picture on Dec. 9, 10, and 11 are:

Dec. 9. Leukocytes—2,800; erythrocytes—3,820,000; hemoglobin—78% Sahli; color index—1.0. Differential count:

	BAS.	EOS.	MYEL.	JUV.	STAB.	SEG.	LYMPH.	MONO.
	-	-	-	-	-	-	100	-
P.M.	Temperature			Pulse		Respiration		
3:00	102.6			122		22		
7:00	100.4			100		24		
9:45	106.0			160		40		
12:00	104.4			110		28		

Transfusion: 500 c.c. citrated blood.

Dec. 10. Leukocytes—1,800; erythrocytes—3,560,000; hemoglobin—68% Sahli; color index—0.9. Differential count:

BAS.	EOS.	MYEL.	JUV.	STAB.	SEG.	LYMPH.	MONO.	
-	-	-	-	-	2	98	-	(Only 50 cells were found on several slides)
A.M.	Temperature			Pulse		Respiration		
4:00	102.0			94		30		
8:00	101.4			104		20		
11:00	99.8			100		22		
P.M.	Temperature			Pulse		Respiration		
3:00	104.2			100		24		
4:00	105.0			132		36		
7:00	101.0			100		24		
12:00	105.4			116		28		

Urinalysis: color, amber; transparency, turbid; specific gravity, 1.016; reaction acid; albumin, 2 plus; sugar, diacetic acid, and indican negative; occasional squamous epithelium, few granular casts.

Transfusion: 500 c.c. citrated blood.

Dec. 11. Leukocytes—1,500; erythrocytes—4,150,000; hemoglobin—85% Sahli; color index—1.0. Differential count:

BAS.	EOS.	MYEL.	JUV.	STAB.	SEG.	LYMPH.	MONO.	
—	—	—	—	—	—	100	—	(Only 50 cells were found for the differential count)
A.M.				Temperature		Pulse		Respiration
4:00.				105.4		106		30
6:30				104.6		112		30
8:30				105.2		94		24
P.M.								
3:00				103.6		128		28
7:00				105.4		180		40

Transfusion: 500 c.c. citrated blood.

Patient expired Dec. 11 at 11:30 P.M.

Autopsy was performed by Dr. Gradwohl. Report follows: The body is that of an aged white woman in good state of nutrition. There are no marks of injury.

The liver is visible three fingers' breadth below the free border of the ribs. Right ovary: very large, $3\frac{1}{2} \times 4$ inches. It is occupied by multilocular cysts. Left ovary: smaller than right; and more of the structure remains. Contains one clear cyst, one chocolate cyst, and coagulated blood. Tubes: normal. Uterus: small, infantile; few intramural fibroids present. Stomach: distended; the extrahepatic biliary passages are open. Right kidney: tunica propia strips without tearing. The fatty capsule is abundant, surface is smooth, stellate veins are engorged, and a cut section shows a wide cortex with fatty changes. Left kidney: same as the right kidney except for considerable scarring. Spleen: small and very firm. A cut section shows that the pulp does not come off on the knife. Trabecular markings are prominent and the spleen is chronically inflamed. Gall bladder: contains thin light colored bile. Liver: enlarged; the cut section shows fatty changes. Pancreas: small.

Upon opening the chest cavity and incising the bone marrow, it is seen to be atrophic; very little red marrow is visible. Marrow taken for microscopic examination.

Lungs: approximate the median line. The right upper lung is somewhat firm, the lower lobe feels like consolidation, the upper lobe is cyanotic and edematous; the lower lobe is hypostatic, and the middle lobe is very edematous. The left lung, upper lobe, is emphysematous. The lower part of the upper lobe is hypostatic and the lower lobe is very red and hypostatic.

Upon opening the pericardial sac it is found to contain about 20 c.c. of clear yellow fluid. There are small petechial hemorrhages on the inside of the parietal pericardium. The left chest contains no free fluid or adhesions. The right chest shows the same. Heart: somewhat fatty with one milk spot on the presenting surface of the left ventricle; right auricle: overdistended with fluid blood and clots; right ventricle: contains fluid blood; left auricle: overdistended with dark clots; left ventricle: empty. Right chamber: somewhat dilated; left chamber: shows a rather thin ventricular wall. Valves: normal. Intima of the aorta: mild degree of arteriosclerosis. Coronary artery: not occluded. There is a mild degree of arteriosclerosis.

Anatomic Diagnosis: (1) Arteriosclerosis of a mild degree; (2) hypostatic pneumonia; (3) fatty degeneration of the liver; (4) cystic ovaries; (5) fibroid tumor of the uterus.

The diagnosis of agranulocytosis cannot be confirmed by anatomic evidence; it must be confirmed by bone marrow examination. The result of these studies will be reported later.

Examination of autopsy tissue:

Tumor of the uterus: pure fibroma.

Left kidney: slight increase in interstitial structures. There is a cloudy swelling in the epithelium of the tubules and a small collection of lymphocytes around a few of the glomeruli.

Heart: definite increase in connective tissue between muscle bundles. There is fibrosis of ventricular wall.

Liver: a great deal of fat between the globules. There is a slight degree of liver cirrhosis. This is a fatty and cirrhotic liver.

Differential counts were made on the bone marrow on two different slides.

This marrow is composed mainly of fat with less than the normal reserve of orthochromatic erythrocytes. Cells are scattered and scarce.

Slide (1)	Neutrophilic myelocytes -----	2%
	Neutrophilic juveniles -----	1%
	Lymphocytes -----	95%
	Orthochromatic normoblasts -----	2%
Slide (2)	Myeloblasts -----	8%
	Promyelocytes -----	4%
	Neutrophilic myelocytes -----	6%
	Neutrophilic juveniles -----	1%
	Lymphocytes -----	77%
	Plasma cells -----	1%
	P. normoblasts -----	3% (1 in karyorrhexis)

There were more cells on the second slide than on the first, but the bone marrow showed less than the usual number of cellular elements.

The Bone Marrow in Agranulocytosis.—The bone marrow shows variable pictures in different types of agranulocytosis. There may be a hyperplastic or aplastic type or a reticuloendothelial type. The usual type is aplastic, which has a serious prognosis. A hyperplastic type probably indicates a favorable outcome. In the aplastic type the bone marrow shows many lymphocytes and plasma cells. In the hyperplastic type, there are numerous myeloblasts and granulocytes in the bone marrow spread. Roberts and Kracke¹ saw degenerative myeloblasts in cases of agranulocytosis reported by them. There is a type of reticuloendothelial change in bone marrow in some cases of agranulocytosis which shows marked hyperplasia of the reticuloendothelial system in addition to the presence of lymphocytes and plasma cells in the bone marrow. These reticuloendothelial cells are clasmatoctytic in appearance.

The blood picture that is typical of this disease shows a differential count of, let us say, 90 per cent lymphocytes and 10 per cent monocytes with no granulocytes whatsoever.

The course of the disease is a leukopenia following the angina; the bone marrow practically ceases to deliver neutrophilic cells. As the disease progresses, the angina develops into gingivitis and tonsillitis and may finally lead to a gangrenous condition. The mortality was previously high, around 90 per cent. With improvement the count rises rapidly, and the patient goes on to recovery. In many cases there are periods of remission with subsequent recurrences.

The Treatment of Agranulocytosis With Penicillin.—

Prior to 1942 the chief aim of physicians in cases of agranulocytosis was to stimulate leukopoiesis by various agents, pentose nucleotide, yellow bone marrow, liver extract, and blood transfusions. These remedies usually proved inadequate and the mortality rate in agranulocytosis remained high.

In 1942 Dameshek and Wolfson² introduced a new policy, that is, not to worry about the bone marrow but to prevent or control the secondary infection; if infection and toxemia can be controlled, the bone marrow may

¹Roberts, S. R. and Kracke, R. R.: *Ann. Int. Med.* 5: 4, 1931.

²Dameshek, William and Wolfson, L. E.: *Am. J. M. Sc.* 203: 819-823, 1942.

take care of itself and recover spontaneously. Thus has developed the rationale for the use of sulfonamides and penicillin. Seven cases of agranulocytosis were successfully treated by sulfonamides but, because sulfonamides themselves may induce agranulocytosis, medical authorities turned to penicillin as an equally effective and safe remedy. It was found that penicillin could be used without harm in the presence of granulocytopenia, or even of agranulocytosis, resulting from severe infections.

One of the most interesting reports on this subject is by Boland, Headley, and Hench.¹ Here they detailed an account of a case of rheumatoid arthritis in which treatment with gold salts resulted in severe agranulocytosis. The total leukocyte count was 3,400 per cu.mm. of blood and no neutrophils were present. With a rapidly progressive folliculitis with marked toxemia, the case appeared very grave. Penicillin therapy was instituted, resulting in a remarkable and dramatic improvement. On one day these physicians thought the patient was dying and the next day he was almost well.

In an entire group of 15 cases, including this case, reported in the literature, the total dosage of penicillin, usually given intramuscularly, varied from 132,000 to 6,480,000 units given within about four to twenty-one days. Recovery within about four to ten days occurred in every case.

It is concluded at this time that penicillin constitutes the most powerful remedy at hand for the prevention or control of secondary infection in agranulocytosis. The mortality rate from this disease, untreated or inadequately treated, has been 70 to 75 per cent; the mortality rate when the disease was treated intensively by leukopoietic agents was still 35 per cent or more. With the added use of penicillin the mortality rate has been reduced tremendously.

More important than the treatment of agranulocytosis is its prevention by avoiding the unnecessary use of drugs which cause this condition. If the drug has to be used, then careful hematologic monitoring is imperative.

Infectious Mononucleosis

Infectious mononucleosis, which has often been confused with acute lymphocytic leukemia, was first reported in the literature by Filatow,² in 1885. Later, in 1889, Pfeiffer³ described it again, calling attention to an infectious process characterized by very definite symptoms, to which he gave the name glandular fever. West⁴ reported an epidemic of ninety-six cases limited to children, with no evidence of throat infection. Tidy and Morley⁵ reported more cases and described the disease again. They noted the enlargement of the axillary and inguinal glands but not the abdominal signs. Tidy⁶ described the epidemic nature of infectious mononucleosis with an incubation period of from 5 to 15 days. Contact infection occurs only in the early stages of the disease, and direct contact or throat droplet infection is probably the mode of transmission. Sprunt and Evans⁷ reported cases of this same disease

¹Boland, E. W., Headley, N. E., and Hench, P. S.: *Proc. Staff Meet., Mayo Clinic* 21: 197-206, 1946.

²Filatow, N.: *Infektionskrankheiten d. Kindesalters*, 1885.

³Pfeiffer, E.: *Jahrb. f. Kinderh.* 29: 257, 1889.

⁴West, J. P.: *Arch. Pediat.* 13: 889, 1896.

⁵Tidy, H. L., and Morley, F. B.: *Brit. M. J.* 1: 452, 1921.

⁶Tidy, H. L.: *Lancet* 2: 180 and 236, 1934.

⁷Sprunt, T., and Evans, F. A.: *Bull. Johns Hopkins Hosp.* 31: 410, 1920.

occurring in young adults with infection of the upper respiratory tract with systemic reaction always present, together with the general glandular enlargement with frequent enlargement of the spleen. All cases showed a lymphocytosis and moderate increase in the total number of white blood cells, together with a blood picture very much like that found in acute leukemia. There was no hemorrhage or severe anemia in any case reported. Longcope⁷ reported cases with acute throat infection but no leukocytosis and very moderate increase in lymphoid cells. Downey and McKinlay⁸ reported nine cases with a study of the hematologic findings. Since 1923 a number of cases have been reported in the literature, together with two contributions, one by Bunnell⁹ and one by Paul and Bunnell,¹⁰ in which they pointed out a definite laboratory test, known as the *heterophile antibody test*, which will be described later.

So far as etiology is concerned, a number of causative organisms have been reported, notably *Listerella monocytogenes*, isolated from rabbits by Murray,¹¹ and *Bacterium monocytogenes* isolated from an apparent case in man by Nyfeld.¹² Other organisms have been described but have not been definitely established. Many believe this to be a virus disease.

Julianelle¹³ also identified *Listerella monocytogenes* which was identified by C. A. Pons from the blood of a patient with infectious mononucleosis. He attempted to determine the significance of *Listerella* in this disease by (a) blood cultures from patients; (b) agglutination tests with the patient's sera; (c) experimental infection in different animals; and (d) stimulation of heterophile antibody by *Listerella* under various conditions. Except for the first culture, all others failed to give any growth. Regarding agglutination tests it was observed in thirteen instances, three times with the "ruminant strain," and ten times with the "rodent strain." He was not able to reproduce in the animals a certain quality to the lymphocytes which is seen in infectious mononucleosis. He did not encounter the so-called Rieder cell in experimental infection. In no instance were the sera found to contain the heterophile antibody despite their high titer for *Listerella monocytogenes*. Julianelle, in other words, concluded that at that time he could not state whether or not infectious mononucleosis of man is an example of *Listerella* infection.

Examination of the biopsy material from lymph nodes of patients with infectious mononucleosis shows that the hyperplasia of lymphocytes is not as extensive as in cases of lymphadenosis. The atypical structure of the lymphocytes and the increase in their number indicate that the disease is due to infection with some organism or virus which has a very specific stimulating effect on the lymphocytes and reticulum and a depressing effect on the granulocytic system.

Blood Picture.—There is no particular change in the number of red cells, the amount of hemoglobin, or the number of blood platelets in infectious mononucleosis. Leukopenia is striking in the beginning. There is usually a

⁷Longcope, W. T.: Am. J. M. Sc. 164: 781, 1922.

⁸Downey, H., and McKinlay, C. A.: Arch. Int. Med. 32: 82, 1923.

⁹Bunnell, W. W.: Am. J. M. Sc. 186: 346, 1933.

¹⁰Paul, J. R., and Bunnell, W. W.: Am. J. M. Sc. 183: 90, 1932.

¹¹Murray, E. G. D., Webb, R. A., and Swann, M. B. R.: J. Path. & Bact. 29: 407, 1926.

¹²Nyfeld, A.: Compt. rend. soc. de Biol. 101: 590, 1929.

¹³Julianelle, L. A.: Ann. Int. Med. 14: 608, 1940.

eukocytosis coincident with enlargement of the lymphatic glands. The differential count shows a high lymphocyte count, often reaching a very high level, with the appearance of immature forms, particularly binucleated cells. (See Plate XXIII.) At this time, the case is almost indistinguishable from lymphocytic leukemia. Downey and McKinlay¹ made an exhaustive hematologic study. They emphasize that the lymphocytosis of infectious mononucleosis has a typical cell which shows varying characteristics in different patients. They divided their patients into three groups, entirely dependent upon the type of lymphocytes present in the blood picture.

The cells of their Type I may be described as follows: cells were not excessively large; they were all atypical, pathologic forms, including even the medium-sized lymphocyte, which has a more basophilic and vacuolated cytoplasm than is usual for these cells. The size and form of the nucleus are of little value in determining genetic relationships or stage of differentiation of the cells, but the quantity and distribution of chromatin and its relation to the parachromatin are of great importance. The characteristic cells are highly differentiated, mature, and are "leukocytoid" lymphocytes, rather than immature "lymphoblasts." The chromatin in the nucleus forms a coarse network of heavy strands and masses which are not sharply separated from the parachromatin. This gives the nucleus a cloudy appearance which is quite characteristic for the lymphocyte. In some cells there is a slight condensation of the chromatin with corresponding separation from the parachromatin. The dense rounded or angular blocks of chromatin characteristic of the nuclei of plasma cells are the result of further condensation. In some of the larger cells, there may be a slight tendency toward a more diffuse arrangement of the chromatin but this is never sufficient to warrant classification of the cell as a lymphoblast or particularly immature cell because the lymphocytic nature of the nucleus always remains evident. The nuclei of the larger cells are frequently placed excentrically and are generally lobulated or indented. The smaller cells also have irregular nuclei. Occasionally, a cell may be found with a very wide and pale cell body consisting mostly of hyaloplasm, but with the nucleus of a mesolymphocyte. The cytoplasm shows a varying degree of basophilia, but most of the cells are very basophilic, much more so than is usual in the normal large lymphocyte or large monocyte. There is an occasional cell with the nuclear and cytoplasmic characters of a plasma cell, but most of them do not have the nuclear structure of plasma cells, although their spongioplasm may be just as basophilic. Frequently there are seen one or more azurophil granules embedded in the hyaloplasm of this region, giving the whole structure the appearance of a centrosphere.

The Type II cases show a nucleus similar to that of the plasma cell. The chromatin strands are very coarse and there are several dense, rounded or angular masses of chromatin among them. This arrangement of chromatin in dense masses is quite characteristic of the nuclei of plasma cells; the nuclei of many of the atypical cells of this series approach the plasma cell type more or less. They have more of a washed appearance. The cytoplasm in Type II is different from that of Type I, it has fewer vacuoles and its spongioplasm

¹Downey, H., and McKinlay, C. A.: *Arch. Int. Med.* 32: 82, 1923,

has a smoother appearance which does not give the foamy, spongy, and stippled effect noted in the cells of Type I. It is generally less basophilic. The nucleus is rarely lobulated.

Type III cases show more leukemic features than any of those described under Types I and II. These Type III cases show large, atypical cells, with vacuolated cytoplasm; in some cells, it is quite basophilic. The leukemic features are particularly interesting. About 1 per cent of the lymphoid cells contains a single large vacuole with an azurophil rod, in addition to smaller vacuoles distributed rather evenly throughout the cytoplasm. The nuclei of some of the atypical cells are dedifferentiated to such an extent that some of them closely approximate the lymphoidocytic type.

In general, the abnormal cell characters in this disease are due to higher differentiation and special cell activity. The peroxidase test on these cells is negative, which would incline one to believe in their lymphocytic origin. Osgood¹ directed particular attention to the nuclear fenestrations in the mature small lymphocytes of this disease. These fenestrations are multiple holes and are considered by Osgood of definite diagnostic importance. The hemograms in Table 63 from a case of infectious mononucleosis illustrate the hematology of the disease.

TABLE 63

DATE	BAS.	EOS.	MYEL.	JUV.	STAB	SEG	LYMPH.	MONO.	IRRITA- TION CELLS
6/19/35	0	1	0	$\frac{1}{2}$	2 $\frac{1}{2}$	25	62 $\frac{1}{2}$	8 $\frac{1}{2}$	0
6/21/35	1	5 $\frac{1}{2}$	0	$\frac{1}{2}$	6	28	73	2 $\frac{1}{2}$	$\frac{1}{2}$
6/22/35	0	1 $\frac{1}{2}$	0	0	1 $\frac{1}{2}$	18	76	2 $\frac{1}{2}$	$\frac{1}{2}$
6/24/35	1 $\frac{1}{2}$	1 $\frac{1}{2}$	0	0	3	27	64	3	0
6/25/35	1	$\frac{1}{2}$	0	0	3 $\frac{1}{2}$	28	62 $\frac{1}{2}$	3 $\frac{1}{2}$	1
6/26/35	0	1 $\frac{1}{2}$	0	0	4 $\frac{1}{2}$	23 $\frac{1}{2}$	65	5 $\frac{1}{2}$	0
7/ 3/35	1	4	0	0	4	35	53	3	0

The Heterophile Antibody Test

Method I.—Owing to the difficulty of making an absolute diagnosis on clinical and cytologic grounds, the suggestion of a serologic diagnostic test, proposed by Paul and Bunnell* in 1932, was hailed with a great deal of satisfaction. This test is based on the presence of sheep heterophile antibodies in the blood of the patient. The heterophile antibody concerned may be defined as an antibody reacting with an agent other than and phylogenetically unrelated to the specific antigen responsible for the production of the antibody in question. The presence of increased sheep heterophile antibodies in the blood in cases of infectious mononucleosis was rapidly confirmed by a number of independent investigators. It is important to know that such antibodies were not increased in those diseases to which infectious mononucleosis so often bears a close resemblance. This test was originally devised by Paul and Bunnell and modified by Stuart and co-workers.† It is performed as follows:

Add 0.5 c.c. of 1 per cent suspension of washed sheep cells to 0.5 c.c. of serial dilutions of the patient's serum. The tubes are incubated at 37° C. for 2 to 4 hours and the degree

*Paul, J. R., and Bunnell, W. W.: *Am. J. M. Sc.* 183: 90, 1932.
†Stuart, C. A., Burgess, A. M., Lawson, H. A., and Wellman, H. E.: *Arch. Int. Med.* 54: 199, 1934.
¹Osgood, E. E.: *Proc. Soc. Exper. Biol. & Med.* 13: 28, 1935.

of agglutination noted. In the routine diagnosis of infectious mononucleosis it is not advisable to incubate the tests overnight in the icebox since, as pointed out by Stuart, Tallman, and Brintzenhoff,[†] many normal sera with little or no sheep cell titer at 37° C. may show a titer as high as 1:160 when tested at 5° C. With confirmatory clinical and cytologic evidence, a titer ranging from 1-80 to 1-10,000 at 37° C. is with few exceptions a positive diagnosis of this disease.

The following technic for the heterophile antibody test adapted by Kracke¹ from Davidsohn, Bernstein, Paul, and Bunnell, has been found more serviceable by us:

Technic.—

Eleven tubes are required for the test, ten tubes for the serial dilutions of the patient's serum, and the eleventh tube for a control over saline and sheep cells used.

To tubes 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 add 0.5 c.c. of physiologic saline; tube 1 receives no saline. Tube number 1 receives 0.5 c.c. of patient's inactivated serum (heated to 55-56° C. for 15 minutes). Tube 2 receives 0.5 c.c. of patient's inactivated serum. The contents of tube 2 are mixed carefully by drawing in and out of the pipette and 0.5 c.c. pipetted to tube 3. Tube 3 is mixed in the same manner, by drawing in and out of a pipette and 0.5 c.c. pipetted to tube 4. Tube 4 is mixed carefully and 0.5 c.c. pipetted to tube 5, and this is continued through tube 10. Tube 10 is mixed and 0.5 c.c. discarded, tube 11 receiving no serum. Dilution of serum in the 10 tubes is now as follows: (1) undiluted; (2) 1:2; (3) 1:4; (4) 1:8; (5) 1:16; (6) 1:32; (7) 1:64; (8) 1:128; (9) 1:256; (10) 1:512. No serum in tube 11. To each of the eleven tubes 0.5 c.c. of a 2% suspension of sheep cells is added. The cells are prepared in physiological saline. (Wash three times immediately before using by adding three times their volume of physiologic saline and centrifuging until firmly packed. The supernatant fluid is removed and the 2% suspension is made by adding 49 volumes of saline.)

After the addition of the sheep cell suspension 1 c.c. of saline is added to each of the eleven tubes. The total quantity in each tube is now 2 c.c.

The tubes are shaken thoroughly and incubated in a water-bath at 38° C. for 1 hour and placed in a refrigerator overnight. One hour at room temperature may be substituted for the refrigeration period, but the test should be checked after refrigeration.

The results are read macroscopically after gently inverting the tubes three times. The results are best reported in degrees. The agglutination is considered as three-plus when the cells remain in a single large clump, as two-plus when the clumps are clearly visible but suspended in clear fluid, and as one-plus when the agglutination can be demonstrated only under a microscope. Always report the strength of the dilution and the degree of the reaction.

The particular usefulness of this test in public health laboratories was reported by Stuart and Mickle.² They state that while this test is diagnostic in a large majority of cases, yet conditions exist under which a serum of moderately high or even very high titer may be erroneously reported positive. Sheep heterophile antibodies similar in character to those found in infectious mononucleosis had been previously demonstrated by Davidsohn³ and many others in the blood of individuals after injections of native or therapeutic horse serum, particularly when serum sickness developed. Davidsohn⁴ has also shown that such antibodies might persist in the blood of a patient for a year or more after treatment with horse serum. Should the serum from such an individual be submitted for an infectious mononucleosis test at any time

[†]Stuart, C. A., Tallman, Juanita, and Brintzenhoff, Esther: *J. Immunol.* 28: 85, 1935.

¹Kracke, R.: *Diseases of the Blood*, ed. 1, Philadelphia, J. B. Lippincott Co.

²Stuart, C. A., and Mickle, F. L.: *Am. J. Pub. Health* 26: 677, 1936.

³Davidsohn, I.: *J. Immunol.* 18: 31, 1930.

⁴Davidsohn, I.: *J. Immunol.* 16: 359, 1929.

previous to the disappearance of the sheep heterophile antibodies associated with the serum sickness, the routine procedure described would be valueless in proving that diagnosis. Stuart, Welch, Cunningham, and Burgess⁵ found that the sheep antibodies in serum sickness were completely adsorbed by emulsions of guinea pig kidney whereas those of infectious mononucleosis were not. Should an apparent infectious mononucleosis case with a history of serum treatment give a high sheep cell titer, it should be titrated as outlined in Method II, below. If the sheep agglutinins are completely or almost completely removed by adsorption with guinea pig kidney, heterophile antibodies of the infectious mononucleosis type are not present. If, on the other hand, the original sheep cell titer of the serum is not significantly reduced by the adsorption, the heterophile agglutinins of infectious mononucleosis are present.

Method II.—The patient's serum should be titrated for antish sheep agglutinin in three ways: (a) unabsorbed, (b) after absorption with guinea pig tissue, and (c) after absorption with beef tissue. If all three titrations are not made, there might be some confusion between the diagnosis of infectious mononucleosis and serum sickness, horse serum reaction, leukemia, Hodgkin's disease, tuberculosis, purpura, and the lymphomas.

Equipment.—

- 24 test tubes, 75 by 12 mm.
- 1 test tube rack
- 4 pipettes, 0.25 c.c.
- 2 pipettes, 0.50 c.c.
- 2 pipettes, 1.0 c.c., graduated in 0.1 c.c.
- 2 sterile syringes, 1 and 2 c.c.
- 2 sterile needles, 20 gauge
- 2 dropper pipettes
- 2 centrifuge tubes, 15 c.c., graduated
- 1 water bath regulated at 56° C.
- 1 centrifuge
- 1 clock or watch
- 1 viewing box such as is used for blood grouping tests

Reagents.—

0.85% Sodium Chloride Solution.

Patient's Serum.—

Obtain in the usual manner, and inactivate in a water bath at 56° C. for 30 minutes.

2.0% Sheep Red Blood Cells.—

Make either from freshly secured blood or prepare from preserved sheep cells.

Place 1.5 c.c. of well-mixed suspension of sheep cells into a 15 c.c. centrifuge tube.

Wash three times in saline or until the supernatant saline is clear and colorless.

This should leave about 0.3 c.c. of packed sheep cells. Dilute 0.3 c.c. of sheep cells with saline to 15 c.c. to make a 2% suspension.

Guinea Pig Kidney Antigen.*—

This can be obtained ready for use.

Beef Cell Antigen.*—

This can be obtained ready for use.

*An excellent preparation of guinea pig kidney antigen and beef red cell antigen is procurable from Certified Blood Donor Service, Jamaica 35, N. Y.

⁵Stuart, C. A., Welch, Henry, Cunningham, John, and Burgess, A. M.: Arch. Int. Med. 58: 512, 1936.

Technic.—**(A) Titration of Unabsorbed Serum.—**

Place 6 test tubes in a test tube rack.

Place 0.4 c.c. of saline in the first tube.

Place 0.25 c.c. of saline in each of the other tubes.

Add 0.1 c.c. of patient's inactivated serum to the first tube.

Mix by drawing into and out of a pipette several times.

Transfer 0.25 c.c. from the first to the second tube, and mix again. (For exact method of handling, see section on blood grouping, page 1015.) Transfer 0.25 c.c. to the next tube and mix.

Continue transferring and mixing through tube 6. This leaves 0.5 c.c. in tube 6. Remove and discard 0.25 c.c. from tube 6.

Add 0.1 c.c. of washed sheep cell suspension to each tube.

Shake well and let stand at room temperature for 2 hours.

Examine for clumping of the red blood cells after gently shaking the tubes.

According to Davidsohn (adapted from Stuart and co-workers), the final dilutions are 1:7, 1:14, 1:28, 1:56, 1:112, etc. (This apparently includes the quantity of sheep cells used: first tube contains 0.05 c.c. serum in a total of 0.35 c.c.)

The titer is the weakest dilution of the serum which gives clumping that is visible to the naked eye.

(B) Titration of Serum Absorbed by Guinea Pig Antigen.—

Shake the suspension of guinea pig antigen thoroughly and place 1 c.c. in a test tube.

Add 0.2 c.c. of inactivated serum.

Shake the tube well, and centrifuge at high speed for 5 minutes.

Transfer the supernatant liquid with a capillary pipette to a clean test tube. This is the absorbed serum.

Place 0.25 c.c. of saline in each of 6 tubes.

Add 0.25 c.c. of absorbed serum to tube 1.

Mix and transfer serially 0.25 c.c. to each tube. Discard the last 0.25 c.c. from tube 6.

Add 0.1 c.c. of washed sheep cells to each tube.

Shake well and let stand at room temperature for 2 hours.

Examine for clumping of the red cells after gently agitating the tubes.

The final dilutions are 1:14, 1:28, 1:56, etc. (includes the added sheep cells).

The titer is the weakest dilution of serum which gives clumping visible to the naked eye.

(C) Titration of Serum Absorbed by Beef Cell Antigen.—

Proceed exactly as for (B), above, using thoroughly shaken beef blood cell antigen in place of the guinea pig antigen.

Interpretation of Results.—

If unabsorbed serum is used, titers below 1:56 are usually of no clinical significance; a titer of 1:56 is suggestive of infectious mononucleosis; titers above 1:56 are indicative of infectious mononucleosis. Since exceptions occur, it is recommended that the differential absorption tests be performed.

Beef cell antigen absorbs the infectious mononucleosis (I.M.) antibody completely, while guinea pig antigen does not. For example, if the titer with unabsorbed serum is 1:56, and the patient has infectious mononucleosis, the titer after beef cell absorption will be 0 or nearly 0, while after guinea pig absorption it will be 1:56, 1:28, or even 1:14, but no lower.

It must be remembered that the specific infectious mononucleosis antibodies are not present throughout the disease, and they should be sought on more than one occasion if the clinical symptoms and the blood findings are suggestive of infectious mononucleosis.

Periodic or Cyclic Neutropenia

There is a group of *chronic* recurrent neutropenias in which no drug or other etiological factor has been found to explain such recurrences. These were described by Reimann,¹ who has been interested in other forms of cyclic disease such as cyclic fevers, and periodic peritonitis. The striking fact about the leukopenia is that it occurs in regular cycles of three to four weeks. Symptomatology is variable and tends to mimic that seen with agranulocytosis, consisting of stomatitis, acute lymphadenopathy, fever, and fatigue. Sore throats and joint pains may be present. Physical findings are not unusual except for the lymphadenopathy and occasional mild splenomegaly. The white blood count varies between 2,000 and 4,000 per cu. mm., with a neutropenia from 0 to 16 per cent.

Primary Splenic Neutropenia

Primary splenic neutropenia was first described in 1942 by Wiseman and Doan.² The outstanding feature of the syndrome is that there is neutropenia associated with splenomegaly. Other symptoms have been quite variable. A fair number of cases have been added to the original number observed by Wiseman and Doan. The clinical picture so far as symptoms and physical findings are concerned is not well defined. The bone marrow is generally hyperplastic. It is our impression that these cases may represent a form of immunohematologic disease in which the leukocyte is a predominantly affected cell.

Acute Infectious Lymphocytosis

Carl H. Smith reported³ an account of a new disease entity, acute infectious lymphocytosis. He reported two cases in 1941 and saw four cases during March, 1943. The fact that three of the patients were members of one family and one a hospital contact impressed him with the possible communicability or infectiousness of the disease. Other cases have been reported at various hospitals in New York City where these first cases were noted.

This is a disease with an elevated leukocyte count persisting for approximately 3 to 5 weeks. The clinical course varies in intensity. This disease can be separated from infectious mononucleosis, acute lymphocytic leukemia, and miscellaneous infections associated with lymphocytosis. The disease has a possible incubation period of 12 to 21 days and may be marked by varying degrees of constitutional reaction at the onset, such as vomiting, irritability, and fever, together with acute abdominal signs and symptoms and occasionally signs of involvement of the nervous system. There is no lymphadenopathy nor is there a palpable spleen. The heterophile agglutination

¹Reimann, H. A.: *Blood* 4: 1109, 1949.

²Wiseman, B. K., and Doan, C. A.: *Ann. Int. Med.* 16: 1097, 1942.

³Smith, Carl H.: *J. A. M. A.*, June 3, 1944; *Lab. Digest* 8: 6, 1944.

reaction is uniformly negative. In two of the cases the biopsy of a lymph node showed strikingly similar microscopic appearance. An outstanding feature in these biopsies was a striking proliferation of the reticuloendothelium of the sinuses, which were blocked by masses of cells. In addition, the lymph follicles showed a number of changes. Some of the germinal centers showed a hyaline degeneration of varying degree. Many of the follicles were notably inconspicuous, and in some sections they were practically obliterated. A feature common to most of the cases was infection of the upper respiratory tract.

The striking feature of these cases was the hyperleukocytosis with relative and absolute lymphocytosis persisting for a prolonged period. Normal small lymphocytes accounted for this increase and presented the most important diagnostic element of the blood picture.

The differential diagnosis from leukemia was based largely on the absence of lymphoblasts. Lymphoblasts are usually round, large, and uniform in size, and show a distinct nuclear pattern. Besides, in acute lymphocytic leukemia with a hyperleukocytosis of the magnitude found in infectious lymphocytosis the blood would show a severe anemia and a decrease in platelets, and enlargement of the spleen and lymph nodes would be noticed.

Examination of the bone marrow also serves to differentiate between leukemia and this disease. In acute lymphocytic leukemia in children, marrow aspiration shows complete replacement of the bone marrow by lymphoblasts. In acute infectious lymphocytosis, on the other hand, the myeloid and erythroblastic elements are present in normal proportions and the only abnormality is the presence of normal lymphocytes in increased percentage.

In the first case, the first blood count showed 45,000 white cells per cubic millimeter, 45 per cent neutrophils, and 48 per cent lymphocytes. In a few hours, the white count rose to 55,400 with 72 per cent lymphocytes. The total count in some cases ran as high as 68,300, and the lymphocytosis amounted to as much as 90 per cent in one case.

The elevated blood levels persist from three to five weeks. The normal lymphocytes in this disease are in sharp contrast to the atypical and abnormal lymphocytic elements in infectious mononucleosis and to the lymphoblasts in leukemia.

The heterophile agglutination reaction was uniformly negative in these cases and the serologic reactions were negative for lymphocytic choriomeningitis and for influenza A and B virus.

In all cases the disease was uncomplicated and had a uniformly favorable outcome.

The evidence presented by Smith clearly indicates that acute infectious lymphocytosis is a heretofore unrecognized communicable disease in which the blood picture serves as an expression of the infection.

The author saw one case of this type in 1942 which puzzled him very much. This report by Smith clarified the situation in his mind regarding whether the case was really a new type of disease or whether it was a simple infectious condition with a concomitant lymphocytosis.

TABLE 64.—MEANS OF DIFFERENTIATING SOME CONDITIONS CHARACTERIZED BY LYMPHOCYTOSIS

	PERTUSSIS	ALBUKEMIC LEUKEMIA	CHRONIC LYMPHO- CYTIC LEUKEMIA	ACUTE LYMPHO- CYTIC LEUKEMIA	ACUTE INFECTIOUS LYMPHO- CYTOSIS	INFECTIOUS MONO- NUCLEOSIS	AGRANULO- CYTOSIS	IDIOPATHIC APLASTIC ANEMIA	PERNICIOUS ANEMIA	ENDOCRINE DISTURB- ANCE	CHRONIC TUBER- CULOSIS
White count	May be 40,000 or higher	Normal or subnormal	Very high; 50,000 and up	Extremely high; up to 1,000,000	Very high; 45,000- 70,000	Normal; or slight leuko- cytosis	Very low; below 2,500; may be below 1,000	Progressively lowered; may be be- low 500	Low; average is around 3,000; may be lower	High nor- mal	High normal
Red count	No signif- icant change	Low	Low	Very low	Usually normal	No signif- icant change	Normal or slightly lowered	Progressively lowered; may be be- low 1,000,000	Low; around 1,500,000 to 3,000,000	No signif- icant change; usually lowered	No signif- icant change
Hemo- globin	No signif- icant change	Low	Low	Very low	No signif- icant change	No signif- icant change	Lowered	Progressively lowered	Low	Often lowered	No signif- icant change
Number of lympho- cytes character- istically present	60-80%	About 60%	As many as 90-100%	Up to 100%	45-90%	Around 60-70%	May be as high as 90-100%	May be nor- mal or up to 100%	Around 40- 50% or higher	About 40- 45%	40-50%
Size of lympho- cytes	Small	Large	Small	Large	Mostly small	Large	Small	Very small	Large	Large	Large; size depends upon amount healing

Pathologic or special forms of lymphocytes	—	Lympho-blasts; Rieder forms; atypical lymphocytes; plasma cells	Lympho-blasts are sometimes found; plasma cells	Lympho-blasts: many are atypical; plasma cells	None	Types I, II, and III special forms seen in this disease; plasma cells	—	—	—	—	—
Pathologic red cells	—	Normo-blasts; polychromasia; other nucleated reds may be seen	Normo-blasts; polychromasia	Normoblasts and other nucleated reds; pathologic forms; polychromasia, etc.	None	None	None	None; as a rule, not even polychromasia	Megaloblasts and megalocytes; macrocytic hyperchromic erythrocytes; polychromasia, etc.	Polychromasia; sometimes basophilic punctation; occasional normoblasts	No significant change
Blood platelets	No significant change	Low	Low	Low or absent	Normal	No significant change	Normal	Low relative and absolute counts; may completely disappear	Low absolute counts	No significant change	No significant change
Bone marrow	—	Lymphatic	Lymphatic	Lymphatic	Normal except for lymphocytosis		“Aplastic” type or packed bone marrow	Fatty degeneration; or infiltration with small lymphocytes	Megaloblastic	—	Left shift

The Lupus Erythematosus ("L.E.") Cell Phenomenon

The often bizarre clinical patterns observed in disseminated lupus erythematosus have made the diagnosis of this disease difficult, especially when the typical skin manifestations are absent. A diagnostic aid was added with the finding of the "L.E." cell in bone marrow by Hargraves and associates.¹ These bone marrow findings were soon verified^{2, 3} and others^{4, 5} were able to find these cells in the peripheral blood of patients with lupus erythematosus. Since the original description of this unusual cell, it has been shown that the serum (or plasma) of patients with lupus erythematosus will reproduce the phenomenon when added to the bone marrow^{4, 6} or the blood^{7, 8} of normal persons or of patients suffering from diseases other than lupus.

These observations led to the conclusion that the abnormal factor was present in plasma and Haserick⁹ identified its association with the gamma globulin fraction. Kurnick and associates¹⁰ have reported that in addition to the specific factor present in Fraction II of L.E. sera, other elements participate in the phenomenon, namely (a) desoxyribonuclease, (b) an inhibitor of desoxyribonuclease, and (c) a factor or factors in Fraction III of normal and L.E. sera. Kurnick and co-workers¹¹ have followed these ideas with determination of the susceptibility of leukocytes from different animal species to alteration by lupus erythematosus serum. These observers found that the leukocytes of the chicken and horse were the most susceptible and that those of the guinea pig and dog were highly susceptible. In all these species, the demonstration of L.E. cell formation was much more striking than when using human leukocytes and they recommend an animal substrate of leukocytes for performing the "L.E." cell test.

Very early in the observations on the "L.E." phenomenon it was realized that the abnormal cells were not dependent on anticoagulant and we were able to demonstrate "L.E." cells in sections,¹² as have others.¹³ We realized in these early observations that the phenomenon was essentially an *in vitro* phenomenon, requiring contact of L.E. serum factors with leukocytes for a period of at least 10 to 15 minutes outside the body. We were not able to demonstrate L.E. cells in blood or bone marrow preparations made *immediately* after withdrawal.

¹Hargraves, M. D., Richmond, B. S., and Morton, R. J.: Proc. Staff Meet., Mayo Clin. 23: 25, 1948.

²Haserick, J. R., and Sundberg, R. D.: J. Invest. Dermat. 11: 209, 1948.

³Morton, R. J.: Thesis, University of Minnesota, February, 1947. Quoted by Haserick and Sundberg.²

⁴Hargraves, M. D.: Proc. Staff Meet., Mayo Clin. 24: 234, 1949.

⁵Sundberg, R. D., and Lick, N. B.: J. Invest. Dermat. 12: 83, 1949.

⁶Haserick, J. R., and Bortz, D. W.: Cleveland Clin. Quart. 16: 158, 1949.

⁷Barnes, S. S., Moffatt, T. W., and Weiss, R. S.: J. Invest. Dermat. 15: 403, 1950.

⁸Barnes, S. S., Moffatt, J. W., Lane, C. W., and Weiss, R. S.: Arch. Dermat. & Syph. 62: 771, 1950.

⁹Haserick, J. R., Lewis, L. A., and Bortz, D. W.: Am. J. M. Sc. 219: 660, 1950.

¹⁰(a) Kurnick, N. B., Parisier, S., Schwartz, L. I., Lee, S. L., and Irvine, W.: J. Clin. Invest. 31: 1036, 1952.

(b) Kurnick, N. B., Schwartz, L. I., Parisier, S., and Lee, S. L.: J. Clin. Invest. 32: 193, 1953.

(c) Kurnick, N. B.: Am. J. Med. 14: 753, 1953.

¹¹Carrera, A. E., Reid, M. V., and Kurnick, N. B.: Blood 9: 1165, 1954.

¹²Barnes, S. S., Moffatt, J. W., Lane, C. W., and Weiss, R. S.: Arch. Dermat. & Syph. 62: 771, 1950 (footnote 10).

¹³Lee, S. L., Michael, S. R., and Vural, I. L.: Am. J. Med. 10: 446, 1951.

The use of guinea pig or dog blood as a substrate is suggested for laboratory use as a means of increasing the sensitivity of the test (see page 830).

The finding of L.E. cells in the peripheral blood or bone marrow of any patient imposes a diagnosis of lupus erythematosus, although a few exceptions to this statement are to be found: individual cases of dermatitis herpetiformis,¹⁴ pernicious anemia,¹⁴ leukemia,¹⁵ multiple myeloma,¹⁶ and primary amyloidosis.¹³ Haserick¹⁷ contaminated plasma samples with fungi and simulated the L.E. cell phenomenon. Walsh and Zimmerman¹⁸ have reported three cases of severe penicillin reaction in which L.E. cells were found in the bone marrow and the plasma factors in the plasma. More recently a lupuslike syndrome has been described in patients treated with hydralazine (Apresoline) for hypertension,¹⁹ in which L.E. cells can be demonstrated. The phenomenon disappears after withdrawal of the drug. Despite these few exceptions, the finding of L.E. cells should be strong evidence of the diagnosis of lupus unless proved otherwise. On the contrary, failure to demonstrate such cells does not militate against such a diagnosis. Our experience has been that L.E. cells are most abundantly present in the acute cases of lupus, and are particularly easy to demonstrate in the terminal phases of the disease. The finding of L.E. cells in patients in remission is variable. At times we have noted inexplicable variations in numbers of L.E. cells. Occasional patients are seen whose preparations contain numerous L.E. cells on one day and only rare forms the following day. We have observed, as have others, so-called precursor forms in some of our preparations. However, we report such forms as only suggestive and do not make a diagnosis of L.E. cells unless they are typical. As with many other laboratory searches, the incidence of positive L.E. preparations is proportional to the vigor with which the pursuit is made, and often many preparations must be made on a given patient before these cells are found. Caution must be exercised in discriminating between erythrophagocytosis by polymorphonuclear leukocytes and L.E. cells. In the former instance, the bodies are the orange or salmon pink of red cells, whereas in the latter, the ingested bodies are a violaceous color with Wright-Giemsa stain.

In unpublished studies in 1950, we originally demonstrated* L.E. cells in sections of bone marrow and performed histochemical studies on this material. In addition to typical L.E. cells, there were found free bodies which showed all of the characteristics of the phagocytized elements. With routine hematoxylin and eosin, the L.E. bodies stained a fuzzy homogeneous lavender blue, whereas normal nuclei stained a dark blue. When only Schiff reagent was applied to this section material, neither normal nuclei nor the bodies took the stain; however, preliminary treatment with hydrochloric acid resulted in brilliant fuchsinophilic staining. In sections treated with periodic acid and Schiff reagent, followed by counterstaining with Mayer's alum hematoxylin, the nuclei of intact cells took the characteristic hematoxylin stain but the L.E.

*Dr. Eli M. Nadel, U. S. P. H. S., carried out the histochemical studies.

¹⁴Berman, L., Axelrod, A. R., Goodman, H. L., and McClaughry, R. I.: *Am. J. Clin. Path.* 20: 403, 1950.

¹⁵Fisher, G. S., and Moyer, J. B.: *Grace Hosp. Bull.* 28: 3, 1950.

¹⁶Montgomery, H., and McCreight, W. G.: *Arch. Dermat. & Syph.* 60: 356, 1949.

¹⁷Haserick, J. R.: *J. Invest. Dermat.* 10: 211, 1951.

¹⁸Walsh, J. R., and Zimmerman, H. J.: *Blood* 8: 65, 1953.

¹⁹Dunstan, H. P., Taylor, R. D., Corcoran, A. C., and Page, I. H.: *J. Lab. & Clin. Med.* 42: 801, 1953.

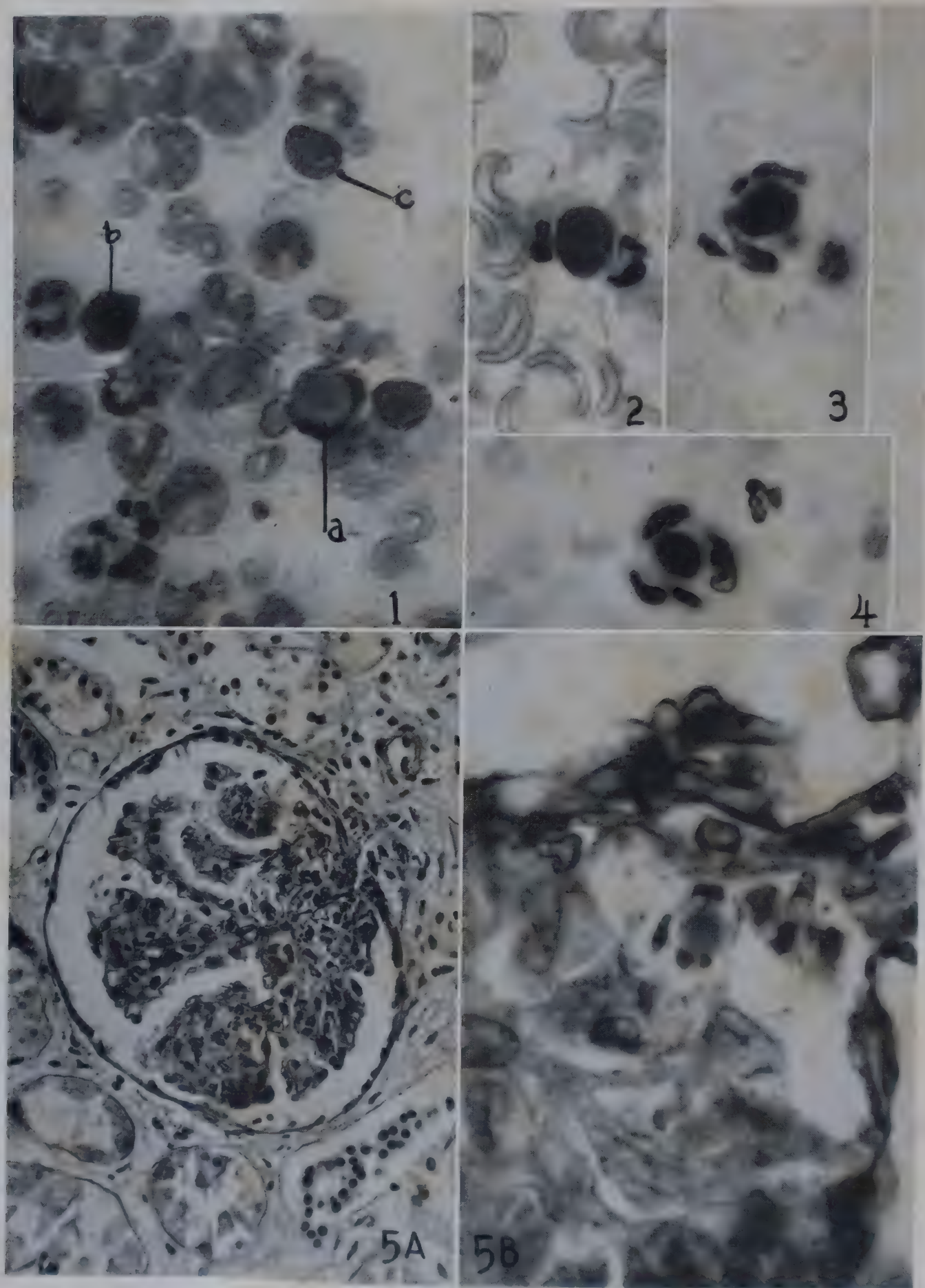


Fig. 198.—Lupus erythematosus ("L.E.") cell phenomenon. Bone marrow spreads and tissue sections. 1, Spread of sternal marrow aspirate. An intracellular (a) and an extracellular (b) L.E. body are seen in addition to a degenerated nucleus (c). (Wright-Giemsa stain; $\times 940$.) 2, Paraffin section of the sternal marrow aspirate with an L.E. cell. (H & E has been treated with ribonuclease and stained with toluidine blue. L.E. body stains similarly to the nuclear material. ($\times 1,530$.) 3, Paraffin section as in 2. Tissue has been stained by the Fuelgen method. L.E. body stains similarly to the nuclear material. ($\times 1,530$.) 4, Paraffin section of kidney of a patient with clinical and autopsy diagnoses of disseminated lupus erythematosus. Sections have been treated with hyaluronidase and stained by the Hotchkiss method. Intra- and extracellular L.E. bodies were seen. ($\times 280$.) 5A, Higher power of 4A, showing a typical L.E. cell. ($\times 1,530$.) (From the collection of H. Agrest.)

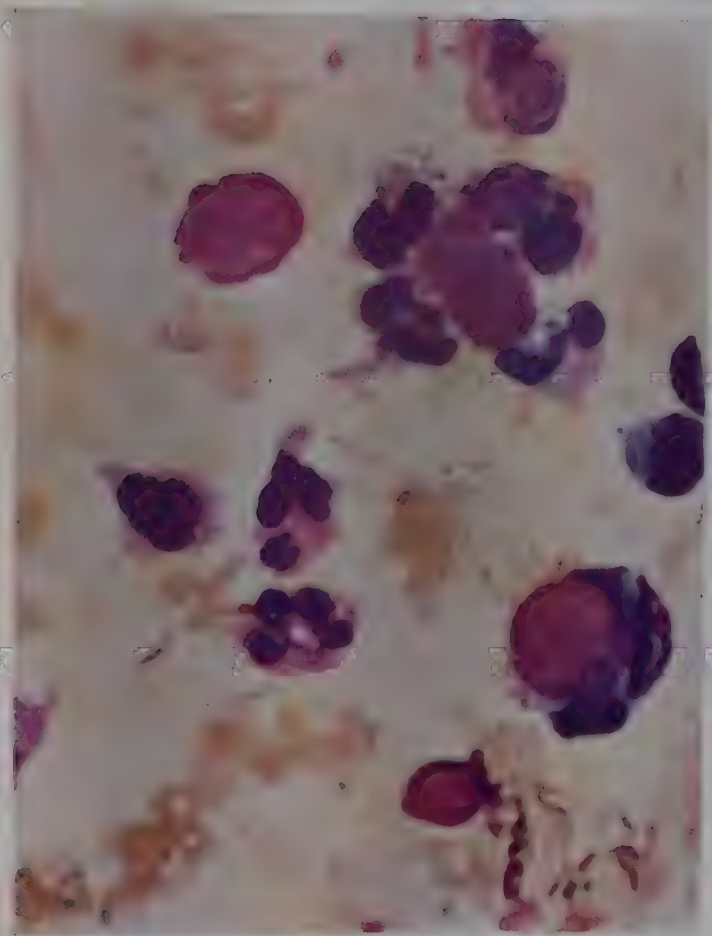


PLATE XXI.—LUPUS ERYTHEMATOSUS ("L.E.") CELLS.

The cluster in the upper right-hand corner is a typical "rosette" with an L.E. body in the center. The cell containing a body, in the lower right-hand corner, is a "tart" cell, which is not pathognomonic of lupus erythematosus. Source of material, bone marrow. $\times 800$.



bodies were homogeneous and a muddy, reddish purple. Neither saliva nor hyaluronidase altered the periodic acid-Schiff positive staining properties of the L.E. bodies. The extracellular and intracellular bodies were negative for iron and for mucin.

From these studies we concluded that the L.E. bodies which we saw in sections were identical to the "hematoxylin staining" bodies first described by Gross²⁰ in tissue sections and in subsequent studies by Genzler and Fox²¹ and Klemperer and associates.²² Cytochemical studies on these hematoxylin bodies²³ were in close correspondence with our studies of kidney displaying hematoxylin bodies. This kidney material was subjected to the same histochemical studies as our bone marrow material; the bodies in the two specimens showed corresponding cytochemical results, a postulate prophesied by Klemperer and co-workers.²³ We suggested at that time that our histochemical studies characterized the L.E. bodies as having approximately 72 per cent nucleoprotein, the percentage suggesting a cell with a relatively large nucleus and a sparse cytoplasm as the source of the L.E. cells. The lymphocyte could well fit this description, its altered nucleus forming the L.E. body. We then visualized the formation of L.E. cells as taking place in two stages. First, there is contact of the abnormal plasma factor with lymphocytes, stripping the cytoplasm of these cells and affecting the nucleus. This phenomenon can occur *in vivo* or *in vitro* and results in a free L.E. *body*. The second stage is the formation of the classical L.E. *cell* brought about by the phagocytosis of this *body* by polymorphonuclear leukocytes. This second phase is an exclusively *in vitro* phenomenon, dependent in part on the time of incubation.

Lee and associates¹³ came to similar conclusions, linking the L.E. cell to hematoxylin-staining bodies. They suggested that in the gamma globulin fraction of lupus plasma, there is a factor capable of altering desoxyribose nucleic acid metabolism. They believed that the inclusion body of the L.E. cell was altered nuclear material derived from polymorphonuclear leukocytes and lymphocytes. Rohn and Bond,²⁴ in supravital studies, found the L.E. inclusion body to be a mature autolyzed neutrophile.

Technic.—

The present method is adapted to routine clinical use.

Collect several c.c. of venous blood or 1 c.c. of bone marrow with an anticoagulant.

Mix thoroughly and allow to stand at room temperature for 1½ to 2 hours. If a 37° C. incubator is available, incubate the tube for 1½ hours.

Centrifuge at 1,500 r.p.m. for 10 minutes.

Aspirate the buffy coat along with a small amount of plasma.

Expel the mixture on a glass slide and touch the edges of successive clean slides to the pool and spread over the surface of slides, shaking these vigorously in the air for rapid drying.

Stain with Wright-Giemsa combination stain.

²⁰Gross, L.: Contributions to the Medical Sciences in Honor of Emanuel Libman, International Press, New York 2: 527, 1932.

²¹Genzler, A. M., and Fox, T. T.: Arch. Int. Med. 65: 26, 1940.

²²Klemperer, P., Pollack, A., and Baehr, G.: Arch. Path. 32: 569, 1941.

²³Klemperer, P., Gueft, B., and Lee, S.: J. Mt. Sinai Hosp. (N. Y.) 16: 61, 1949.

²⁴Rohn, R. J., and Bond, W. H.: Am. J. Med. 12: 422, 1952.

Demonstration of L.E. Cells, Method of Schultz et al.

Schultz and co-workers¹ have devised an ingenious microtechnic for demonstrating the L.E. cell phenomenon.

Place 2 or 3 drops of blood from a syringe or a finger puncture on a clean glass slide in 3 places on each slide.

Allow to clot.

Incubate at 37° C. for 20 minutes in a closed Petri dish containing moistened filter paper.

Wash the slide with a fine spray of physiologic saline to remove most of the clot, leaving thin "disks" adherent to the slide. Microscopic examination of these disks shows them to consist of polymorphonuclear leukocytes almost exclusively.

Immediately following the washing process, add several drops of the test serum or plasma to each disk, and place a cover slip over each preparation.

Place the slides again in covered Petri dishes containing moistened filter paper, and incubate at 37° C. for 1 hour.

Remove the cover slips and allow the slides to dry in the air by standing them on edge so that the excess fluid drains off.

Stain the slides by the Wright-Giemsa stain (or some modification of the Romanowsky stain), using neutral distilled water.

Scan under low-power magnification of the microscope for individual L.E. cells or for clumping of leukocytes with rosette formation.

If the specimens have been properly prepared, there will be leukocytes with normal morphologic characteristics other than those changes associated with the L.E. cell phenomenon.

If the leukocytes appear abnormal and the test is negative, there is doubt concerning the validity of the test on technical grounds, and the procedure should be repeated.

Schultz and associates recommend that a minimum of six disks be examined before considering the test negative. They state that the disk method was positive in some instances where other methods were negative, and that this method showed "either as many L.E. cells per high-power field as two other commonly used tests or more than the number seen by these methods."

Epidemic Hemorrhagic Fever

In June, 1951, United Nations Troops in Korea encountered an acute disease characterized by fever, prostration, vomiting, a variety of hemorrhagic manifestations, shock, proteinuria, and renal failure. A disease known to the Japanese as epidemic hemorrhagic fever and to the Russians as epidemic hemorrhagic nephroso-nephritis or Far Eastern hemorrhagic fever has been described in two U. S. Army Technical Bulletins on Korea and Manchuria.²

This disease was first described by the Russians who encountered it in Far Eastern Siberia in the middle 1930's. They found that the incubation period was generally ten to fifteen days, a single attack conferred immunity, and that the disease-producing agent would pass through a size N Berkefeld filter. It has been impossible to reproduce this disease in animals. In spite of the failure of animal experiments, it is suggested that hemorrhagic fever

¹Schultz, I., Baum, J., and Ziff, M.: *Proc. Soc. Exper. Biol. & Med.* 88: 300, 1955.

²TB MED 208, 1945 and TB MED 216, 1946.

may be transmitted to man by rodent ectoparasites, such as fleas, mites, ticks, and chiggers. These chiggers seem best to fit the observed epidemiologic facts.

The complete story on this interesting and new disease is found in the Symposium on Epidemic Hemorrhagic Fever by various authors.² Briefly, the clinical picture is characterized by sudden onset, in a person recently situated in an endemic area, of chills, fever, prostration, frontal headache, marked thirst, and myalgias. There is a marked facial flush, injection of the palate and conjunctivae, and petechiae of the conjunctivae, palate, axillary folds, and waistline. Proteinuria and reduction in specific gravity of the urine are present.

This clinical course may be divided into four phases, each designated by a characteristic physiologic aberration: (1) febrile, (2) hypotensive, (3) oliguric, and (4) diuretic.

In this Symposium, Lt. Robert J. Lukes describes the pathology of 39 fatal cases of this disease. There was almost constant occurrence of hemorrhage in the right atrium of the heart, of severe congestion, hemorrhage, and infarctlike necrosis in the renal medulla, and hemorrhage and necrosis in the anterior lobe of the pituitary gland, in association with the pattern of the vascular changes. All this supports the contention proposed in the Japanese literature and by Steer and Hüllinghorst that capillary damage is the basic process. Retroperitoneal edema, observed only once in secondary shock, is the striking pathologic feature when the patient dies during shock in early phases of the disease. The abrupt increase in incidence of pulmonary edema in the oliguric phase, particularly after the eighth day of the disease when retroperitoneal edema is rare, supports the clinically suspected role of redistribution of fluid and electrolytes with resorption of the abnormal interstitial fluid.

The frequent coexistence of pulmonary edema and bronchopneumonia in the oliguric and diuretic groups and the high incidence of pulmonary abscess formation support the concept of Moon that pulmonary edema of the albuminous type is conducive to the development of pulmonary infections.

The presence of necrosis of the anterior lobe of the pituitary gland in cases suggests that shock is not the only major factor in the pathogenesis of pituitary necrosis in hemorrhagic disease. Anoxemia is also an important factor, and is probably the major cause of necrosis in the absence of shock.

Studies of the coagulation mechanism in epidemic hemorrhagic fever reveal abnormalities in prothrombin activity, clotting time, and platelet count. Persistence of thrombocytopenia, which develops early in the disease, through the oliguric-hypertension phase, would appear to be causatively related to the bleeding phenomenon observed. Occasional marked deficiencies in plasma coagulation factors are found in seriously ill patients and may contribute to clinically significant bleeding. This plasma coagulation defect is similar to that observed in acute renal insufficiency and has not been clearly defined.

Studies by Giles and Langdon in this report suggest that some mechanism other than decrease in total blood volume is a primary cause of shock in

²Am. J. Med., May, 1954.

hemorrhagic fever. Although most patients showed normal red cell volumes during the febrile and shock phases of the disease, two showed a significant decrease during severe shock, and many others have decreases in other phases.

Studies by Froeb and McDowell in this report in the *American Journal of Medicine* on renal function in hemorrhagic fever show that the ability to form urine of high specific gravity may persist after the initial decrease in C_{IN} and C_{PAH} but is subsequently lost. The clearance returns to normal before the concentrating ability.

The following electrolyte abnormalities were observed during the course of hemorrhagic fever: (1) During the oliguric phase, hyponatremia, hyperkalemia, hypocalcemia, and retention of anions were common. However, serious hyperkalemia was rarely encountered and acidosis was not severe. (2) During diuresis, hyponatremia occasionally resulted from a "salt-losing" necropathy but more often was due to unknown causes. The hyperkalemia frequently increased during the early diuresis but at this time was not dangerous. Potassium deficiency, associated with the usual symptoms and signs of hypokalemia, sometimes occurred even though hypokalemia was not present. Hunter thus describes electrolyte abnormalities in hemorrhagic fever.

SUMMARY OF THE CHANGES IN THE BLOOD PICTURE

The following summary offers to the reader a recapitulation of the changes in the Schilling differential count seen in various pathologic conditions.*

I. Leukocytes With Increase in Basophiles.

1. Myelocytic leukemia.
2. Polycythemia vera.
3. Serum injection.
4. Hemophilia.
5. Convalescence from beriberi.
6. After multiple blood transfusions
7. In many conditions customarily accompanied by eosinophilia

II. Eosinophilia With Increased White Count.

Note: It is a biologic rule that severe irritations do not cause weaker or paradoxical reactions; that is, the same processes which in moderate degrees may produce eosinophilia, in greater degrees cause either hypereosinophilia or aneosinophilia.

1. Exudative processes: bronchial asthma, exudative diathesis and exudative intestinal catarrh of children, colitis membranacea, Quincke's edema, etc.
2. Anaphylaxis and anaphylactoid conditions: serum disease, hay fever, urticaria, migraine, idiosyncrasies, etc., irritations of vagus nerve.
3. All diseases due to worms wherever located. (Eosinophilia may dwindle to aneosinophilia [insufficiency] because of overinfestation.)

Trichinosis, schistosomiasis hepatica, ancylostomiasis, bilharziasis, filariasis, *Diphyllbothrium latum* infestation, echinococci, and intermittently with all intestinal parasites.

4. Skin diseases: psoriasis, pemphigus, urticaria, eczema, herpes zoster, impetigo contagiosa, some dermatitides, prurigo hebrae, pre-mykotic exanthema, mycosis fungoides, purpura.
5. Scarlet fever beginning on the third day of the eruption.
6. Gonorrhea, especially in females.

*Some of the hemograms given here are from Schilling: *The Blood Picture* (Translator, Gradwohl), The C. V. Mosby Co.

7. Tumors, occasionally, especially those of the lungs and peritoneum; rectal carcinoma, especially in serous metastases.
8. Nephrosis or nephritis occasionally (slight affections).
9. Muscular rheumatism (Bittorf).
10. Leukemia. This serves to differentiate leukemia from leukemoid pictures.
11. Pernicious anemia in the beginning, during remissions, under liver therapy.
12. Convalescence from infections (good sign).

III. Decrease or Absence of Eosinophiles.

THIS IS CONSIDERED AN UNFAVORABLE SYMPTOM.

1. Overirritation of eosinophilic processes, particularly in severe affections.
 - a. Due to worms.
 - b. Height of serum disease.
 - c. Pernicious anemia in severe stages.

Examples of Eosinophilia

	LEUKO-CYTES	BAS.	EOS.	MYEL.	JUV.	STAB.	SEG.	LYMPH.	MONO.	REMARKS
Bronchial asthma	14,000	1	10	—	—	3	40	43	3	
Hay fever	—	1	6	—	—	3	51	32	7	
Trichinosis	11,000	$\frac{1}{2}$	57 $\frac{1}{2}$	1	1	9	14	15	1 $\frac{1}{2}$	$\frac{1}{2}\%$ plasma cells
Trichuris trichiura infestation	8,400	$\frac{1}{2}$	11	—	2	14	44	18	5	
Pemphigus filiac	Low	1	12	—	1	5	48	24	9	
Eczema	Normal	1	19	—	—	—	48	18	14	
Scarlet fever—3rd day	11,760	—	5	—	5	18 $\frac{1}{2}$	49 $\frac{1}{2}$	16	6	$\frac{1}{2}\%$ plasma cells
Peritoneal carcinoma	40,000	—	19	—	28	6	38	4	5	
Glomerulonephritis	—	—	8 $\frac{1}{2}$	—	—	3	52	27 $\frac{1}{2}$	9	
Purpura rheumatica	—	1	14	—	—	10	39	27	9	
Amebic dysentery	6,500	1	5	—	—	6	56	31	1	Amebae in the stools

Course of Eosinophiles in Infection

	LEUKOCYTES	BAS.	EOS.	MYEL.	JUV.	STAB.	SEG.	LYMPH.	MONO.
Lobar pneumonia	12,000	—	—	—	4	32	51 $\frac{1}{2}$	11 $\frac{1}{2}$	1
	8,200	—	(+)*	—	3	22	63	10 $\frac{1}{2}$	1 $\frac{1}{2}$
	8,100	—	3 $\frac{1}{2}$	—	1 $\frac{1}{2}$	21	44 $\frac{1}{2}$	23 $\frac{1}{2}$	6
	6,900	$\frac{1}{2}$	4	—	$\frac{1}{2}$	8	50	22	5

*Present in the thick drop.

- d. Malignant granuloma when severe.
- e. Acute leukemias, severe stages.
- f. Aleukemia, severe stages.
- g. Agranulocytosis, severe stages.
2. Acute stages of infections and most infectious diseases with the exception of gonorrhea and scarlet fever: particularly typhoid fever, pneumonia, diphtheria, bacillary dysentery, relapsing fever, measles.
3. Protozoal diseases during attacks.
4. Uremia and other intoxications.
5. Marked drop (50%) following administration of ACTH or cortisone.

IV. Normal Total Neutrophiles and Normal White Blood Count.

- 1. Normal individuals in the morning after a night's fast.
- 2. Diseases that very rarely influence neutrophiles.

V. Normal Total Neutrophiles and Low White Blood Count.

- 1. Severe pernicious anemia. (This usually shows neutropenia.)
- 2. Severe aplastic anemia. (This usually shows neutropenia.)
- 3. Osteoscleroses and diseases with reduction of bone marrow.

VI. Neutrophilia Without Shift But With Increased White Blood Count.

- 1. During digestion.
- 2. Muscular activity.
- 3. Spasmodic conditions.
- 4. After hemorrhages.
- 5. Polycythemia (at times a slight shift).
- 6. Very superficial or benign infections.
- 7. Simple intestinal catarrh.
- 8. Nephritides.
- 9. Abortive cases of infectious disease.
- 10. Polyarthrititis acuta. (Often shows a right shift.) (An acute infection without a left shift.)
- 11. Tetanus.
- 12. Struma.
- 13. Uncomplicated, malignant tumors.
- 14. After sodium chloride infusions, temporarily.
- 15. After certain medicaments.
- 16. During violent internal or external hemorrhages.

VII. Neutrophilia With Slight Stab Shift With High Normal or Increased White Blood Count.

- 1. Mild cases of acute infections and protozoal diseases: anginas, catarrhs, malaria during attacks.
- 2. Severe diseases of the digestive system: intestinal catarrhs, liver diseases, including jaundice, where the epithelia are alone involved.
- 3. Superficial, benign, or encapsulated infections:
 - a. After operations.
 - b. After incision of abscesses.
 - c. Suppurative wounds without general involvement.
 - d. Slight catarrhal appendicitis.
 - e. Open suppurations of eyes, ears, pharynx.
 - f. Appendicitis, gynecologic, and liver pus collections, entirely encapsulated.
 - g. Chronic diseases with neutrophilic irritation (septicemias and endocarditis).
 - h. Broken down ulcerative tumors.
 - i. Mild active tuberculosis.
 - j. Tuberculous abscess formation.

THESE SLIGHT SHIFTS ARE IMPORTANT FOR THE FOLLOWING REASONS:

- 1. To determine infectious processes.
- 2. To estimate the latent continuance of apparently healed infections, or the danger of relapse.
- 3. To outline as relatively harmless acute illnesses or exacerbations of severe conditions which have been reactivated.

Examples of Neutrophilia Without a Shift

	LEUKOCYTES	BAS.	EOS.	MYEL.	JUV.	STAB.	SEG.	LYMPH.	MONO.
Nephritis	18,000	—	2	—	—	4	76	13	5
Polyarthrititis acuta	26,750	—	—	—	—	3	85	10	2

Examples of Neutrophilia With Simple "Stab" Shift

	LEUKO-CYTES	BAS.	EOS.	MYEL.	JUV.	STAB.	SEG.	LYMPH.	MONO.	
Catarrhal jaundice	6,500	1	4½	—	½	9½	59½	17	8	Large wound
Burn	23,000	—	—	—	—	10	66	18	6	
Encapsulated abscess of kidney	9,300	—	½	—	—	11½	63½	20½	4	
Benign appendicitis	7,500	1	4	—	—	7	53	27	8	
Tubercular pleuritis	7,050	—	2	—	—	6	72	14	6	

VIII. Neutrophilia With Regenerative Shift and Increased White Blood Count.

1. Very violent exercise (marathon races).
2. Diabetic coma.
3. Pregnancy.
4. Severe cases of many acute infectious diseases: pneumonia, senile pneumonia, diphtheria, scarlet fever, cholera, bacterial dysentery, erysipelas, meningitis epidemica, typhus, variola.
5. Acute protozoal disease during attacks.
6. All acute and progressive internal infections: beginning empyema, newly formed abscess, appendicitis destructiva et perforata, peritonitis, purulent inflammation of bile ducts and urinary passages, pus infection of bone marrow, encapsulated malignant pustular affections of ears, eyes, and pharyngeal region, fresh cerebral suppurations, gynecologic processes, eclampsia, puerperal fever, severe intoxications due to bacterial poisons, heavy metals (lead, sublimate, etc.), coal gas, Lysol, phenylhydrazine.
7. Subsequent effects of leukopenic infection.
8. Acute exacerbations of chronic infections: tuberculosis, and endocarditis.
9. Transitions of leukopenic infection into one with severe regenerative complication: grippe with pneumonia.

Examples of Pregnancy-Labor Leukocytoses

	LEUKO-CYTES	BAS.	EOS.	MYEL.	JUV.	STAB.	SEG.	LYMPH.	MONO.	PLASMA CELLS
4th month		0.2	1.3	—	1.2	11.3	61.6	20.7	3.3	0.4
5th month		0.3	2.0	—	1.1	8.7	63.2	20.7	3.3	0.7
6th month		0.3	2.4	—	2.6	11.2	53.9	25.7	3.8	0.1
7th month		0.1	2.1	—	1.5	12.5	55.4	22.9	5.0	0.5
8th month		0.3	1.2	—	1.4	11.3	57.9	22.4	5.1	0.4
9th month		—	1.8	—	1.9	11.4	55.7	24.4	4.7	0.1
Before delivery	10,930	0.3	2.2	—	1.2	8.7	53.8	22.9	4.9	0.5
During labor	15,635	0.2	0.4	—	2.5	15.0	58.6	18.2	4.5	0.6

Examples of Neutrophilia and Regenerative Shift

	LEUKOCYTES	BAS.	EOS.	MYEL.	JUV.	STAB.	SEG.	LYMPH.	MONO.
Shiga dysentery	6,100	—	1	—	2	21½	57½	14	4
Typhus, 6th day	Normal	—	1	—	9	22	52	11	5
Typhus, before the crisis	Normal	—	(+)	—	5	16	46	17	16
Lobar pneumonia:	24,800	—	—	1	11	9	67	4	8
3rd day	29,500	—	(+)	—	3	26	47	18	6
Senile pneumonia:	Sl. increase	—	—	—	20	17	41	13	9
2d day	8,600	—	—	1	30	18	35	11	5
Grippe pneumonia	5,325	—	½	—	1	8½	70½	13	6½
Tertian malaria during the attack	6,300	—	1	—	10	15	40	20	14
Tropical malaria during the attack	Increased	—	—	—	32	19	10	25	14
Liver abscess before operation	14,000	—	—	—	9	31	38	17	5

Example of Transition From Grippe to Pneumonia

	LEUKOCYTES	BAS.	EOS.	MYEL.	JUV.	STAB.	SEG.	LYMPH.	MONO.
Simple grippe	4,200	—	2	—	$\frac{1}{2}$	11 $\frac{1}{2}$	44	34	8
After several days of fever—pneumonia	4,600	—	—	1	26 $\frac{1}{2}$	11 $\frac{1}{2}$	49	6 $\frac{1}{2}$	5 $\frac{1}{2}$
Second day	7,500	—	—	$\frac{1}{2}$	23	10 $\frac{1}{2}$	58	4 $\frac{1}{2}$	3 $\frac{1}{2}$
4th day	20,000	—	—	—	11	18	61	10	—
5th day	26,860	—	—	—	7 $\frac{1}{2}$	10	75 $\frac{1}{2}$	5	2
6th day	20,000	—	$\frac{1}{2}$	—	1	15 $\frac{1}{2}$	74 $\frac{1}{2}$	4	4 $\frac{1}{2}$
8th day	15,000	—	$\frac{1}{2}$	—	1 $\frac{1}{2}$	9	68	14	7

IX. Neutrophilia With Hyperregenerative Shift With Decreasing or Low White Blood Count and Absolute Aneosinophilia.

THIS IS USUALLY AN AGONAL SYMPTOM OF VERY SERIOUS SIGNIFICANCE

- 1. Very severe infections.
- 2. Perforation peritonitis.
- 3. Croupous pneumonia.
- 4. Malaria comatosa.
- 5. Fatal blackwater fever.

X. Liberation Leukocytosis (with involvement of erythrocytes).

- 1. Complicated bone fractures.
- 2. Metastases of tumors.
- 3. Lymphogranulomata.
- 4. After intravenous injections and transfusions with bad reaction.
- 5. Blood crises, particularly in pernicious anemia, hemolysis, and blackwater fever.
- 6. Nonleukemic myelosis (aleukemic myelosis).
- 7. Marble disease (Albers-Schoenberg's disease).
- 8. Simple myelogenous metaplasia of the spleen.

XI. Persisting Leukemoid Leukocytosis.

- 1. Persisting direct irritation of bone marrow:
 - a. Carcinosis.
 - b. Malignant granuloses.
 - c. Polycythemia vera with hyperplasia of erythropoiesis.
- 2. Severe anemias complicated with infection.
- 3. Doubtful acute myelocytic leukemia.
- 4. Improved stages of agranulocytosis during cure (penicillin).

Examples of Neutrophilia, Leukocytosis, and Hyperregenerative Shift

	LEUKOCYTES	BAS.	EOS.	MYEL.	JUV.	STAB.	SEG.	LYMPH.	MONO.	REMARKS
Empyema of gall-bladder	Very high	—	(+)	±	15	34	34	8	9	
Eclampsia	Very high	—	(+)	1	29	20	34	5	11	
Mercuric chloride poisoning	27,000	—	—	1	9	20 $\frac{1}{2}$	59	5 $\frac{1}{2}$	5	3 hours after taking tablets
Lysol poisoning:										
After 2 hours	60,700	—	$\frac{1}{2}$	1 $\frac{1}{2}$	22	27 $\frac{1}{2}$	37 $\frac{1}{2}$	7	4	
After 4 hours	70,000	—	—	1 $\frac{1}{2}$	16 $\frac{1}{2}$	29	41	8	5	
After 20 hours	Fewer	—	—	7	42	26	14 $\frac{1}{2}$	6 $\frac{1}{2}$	4	Death 9 hours later
Coal gas poisoning	11,000	—	—	—	—	33	45	15	7	Severe shift

Examples of Liberation Leukocytosis With Involvement of the Erythrocytes

	LEUKOCYTES	BAS.	EOS.	MYEL.	JUV.	STAB	SEG.	LYMPH.	MONO.	REMARKS
Carcinosis (metastases in bone marrow)	13,300	—	$\frac{1}{2}$	4	10	25	21	19	20	14 normoblasts, $5\frac{1}{2}$ megablasts, P.+++
Sarcomatosis (metastases in lungs and liver)	High	—	—	2	8	28	51	7	4	Many normoblasts, P.++
Infection of bone marrow	80,000	—	$\frac{1}{2}$	$2\frac{1}{2}$	$28\frac{1}{2}$	$13\frac{1}{2}$	29	$7\frac{1}{2}$	$18\frac{1}{2}$	10 normoblasts to 100 leukocytes

Examples of Fatal Pictures—Severe Left Shift With Moderate Leukocytosis

	LEUKOCYTES	BAS.	EOS.	MYEL.	JUV.	STAB	SEG.	LYMPH.	MONO.	
Septicemia	15,000	—	—	2	26	19	38	8	7	
Dysentery	10,700	—	—	3	25	18	32	$4\frac{1}{2}$	$7\frac{1}{2}$	
Hypostatic pneumonia	12,250	—	—	$1\frac{1}{2}$	4	$20\frac{1}{2}$	$61\frac{1}{2}$	$7\frac{1}{2}$	5	

XII. Neutrophilia With Degenerative Shift With High Normal White Blood Count.

1. Mixed cases (typhoid with sepsis; tuberculosis with secondary infection).
2. Chronic or less virulent infections (hepatic abscess, recurrence, endocarditis).

XIII. Neutropenia With Low White Blood Count and Marked Stab Shift.

1. Kala-azar.
2. Other protozoal diseases *after* attacks.
3. During uncomplicated typhoid and paratyphoid fever.
4. Parotitis epidemica, in severe cases.
5. Pappataci fever.
6. Brucellosis (this sometimes shows neutrophilia).
7. Grippe.
8. Dengue.
9. Not infrequently in tuberculosis.

XIV. Neutropenia With Low White Blood Count and No Known Shift.

1. Pellagra.
2. Yellow fever.
3. Spotted fever.
4. Kedani or tsutsugamushi (scrub typhus).
5. Chicken pox.
6. Poliomyelitis acuta.
7. Ophthalmia sympathica.
8. Pernicious anemia.

XV. Neutropenia With Low Total White Blood Count and No Shift, Often Total Absence of Neutrophiles.

1. Agranulocytosis.
2. Aplastic anemia.
3. Experimentally in anaphylaxis.

XVI. Neutropenia With Shift to the Right.

THESE PICTURES SHOW ABSENCE OR DIMINUTION OF STABS AND NUMEROUS HIGHLY SEGMENTED NEUTROPHILES WITH SIX OR MORE SEGMENTS AND DENSE GRANULES, AND A TENDENCY TO LARGE, OVOID CELLS.

1. Pernicious anemia.
2. Very severe anemias with pernicious blood picture.

- a. Syphilis.
- b. Pernicious anemia of pregnancy.
- c. Sprue.
- d. Carcinoma of the stomach.
- e. *Diphyllobothrium latum* infestation.
- f. Less frequently in other worm diseases.
- g. Occasionally in malaria and septic processes.
- h. Less marked in avitaminoses (pellagra).
- i. Conditions due to insufficient food.
- j. Cirrhosis of the liver.

XVII. Normal Lymphocytes and Normal White Blood Count.

- 1. In normal individuals after a night's fast.
- 2. Diseases not influencing the lymphocytic picture.

Examples of Leukopenia With Neutropenia and Degenerative Shift, With Lymphocytosis and Monocytosis

	LEUKOCYTES	BAS.	EOS.	MYEL.	JUV.	STAB	SEG.	LYMPH.	MONO.	REMARKS
MILD typhoid fever	3,000	—	—	—	—	30	20	38	12	Neutrophilia
Typhoid fever	4,000	—	—	—	—	23	11	54	12	
Malta fever	2,325	1	1	—	—	21	28	41	8	
Malta fever	6,000	—	—	—	—	7	39	30	24	
Malta fever	2,510	1	1	±	1	19	58	14	6	
Simple grippe	3,025	—	2	—	—	9	36	43	10	
Simple grippe	4,900	—	1	±	1	4½	51½	28	8	
Simple grippe	Low	—	2	—	—	8	36	42	12	

Example of Agranulocytosis (Patient Recovered)

	LEUKOCYTES	BAS.	EOS.	MYEL.	JUV.	STAB	SEG.	LYMPH.	MONO.	REMARKS
Jan. 11	1,600	—	(—)	—	—	1	1	72	26	5 promyelocytes
Jan. 13	1,600	—	(—)	—	2	7	3	66	22	
Jan. 19	4,600	—	(—)	10	13	3	12	41	16	
Jan. 22	8,960	—	(—)	17	16	6	21	31	9	
Jan. 31	High	—	(—)	—	—	4	56	30	10	

Examples of Neutropenia With Right Shift

	LEUKOCYTES	BAS.	EOS.	MYEL.	JUV.	STAB	SEG.	LYMPH.	MONO.	REMARKS
Pernicious anemia	3,200	—	3½	—	—	½	37	56	3	R.B.C.— 1,392,000; hgb. 36%; C.I.—1.38
Same case two months later	1,300	—	1	—	—	1	21	76	1	R.B.C.— 1,000,000; C.I.—1.2

- 3. Physiologic leukocytoses and benign infectious leukocytoses of long duration, as a favorable symptom.
- 4. In association with aplastic or aregenerative anemias.

XVIII. Lymphocytosis With Increased Total White Count.

- 1. Constitutional in some individuals.
- 2. First phase of physiologic or very slight irritation leukocytosis, during exercise, digestion, etc.
- 3. Epilepsy during the attacks.
- 4. Lymphocytosis of crying nursing infants.
- 5. Parotitis epidemica during course of mild cases.

6. Pertussis.
7. Uncomplicated benign (afebrile) tuberculosis and syphilitic processes.
8. After certain medicaments; as Adrenalin.
9. Lymphocytic leukemia.
- 10..Acute infectious lymphocytosis.
11. Infectious mononucleosis.

XIX. Lymphocytosis With Normal or Low White Blood Count.

1. All conditions with neutropenia as an associated condition.
2. Lymphocytic aleukemia.
3. Granulomatoses, especially in stages without fever.
4. Neurasthenia and vagotonia (constitutional).
5. Basedow's disease.
6. Acromegaly.
7. Myxedema.
8. Aregenerative anemias, particularly pernicious anemia.
9. Most protozoal diseases during recurrence.
10. During attacks or intervals of conditions caused by viruses.
11. Beriberi.
12. Pure scurvy.
13. Metabolic diseases in chronic stages, similar to conditions of hunger.
14. Achylia gastrica simplex.
15. Uncomplicated benign tuberculosis.
16. Uncomplicated syphilis.
17. Continued roentgen exposure.
18. After infections, as a phase of recovery.
19. After vaccinations (typhoid, cholera, dysentery, tuberculin, etc.)

XX. Lymphocytosis With Marked Low Total White Blood Count.

1. Grippe.
2. Pappataci fever.
3. Typhoid fever.
4. Brucellosis.
5. Kala-azar.
6. Agranulocytosis.
7. Aleukia.
8. Aplastic anemia.
9. Pernicious anemia.

Examples of Absolute Lymphocytoses

	LEUKOCYTES	BAS.	EOS.	MYEL.	JUV.	STAB	SEG.	LYMPH	MONO.
Tuberculosis	8,300	—	1	—	—	5	40	42	12
Chronic arthritis deformans	10,600	—	2½	—	—	6	30	55½	6
Lymphatic leukemia	247,700	½	2	—	1½	5½	5	83	2½
Basedow struma	Normal	—	7	—	1	3½	44	36½	8
Chronic malaria	Normal	—	1	—	—	7	49	36	7
Chronic amebic dysentery	—	—	6	—	1	2	32	51	8
Beriberi	5,900	2	3	—	—	5	36	46	8
Scurvy and purpura	5,100	—	3	—	—	2	42	47	6
Roentgenologist	6,000	—	13	—	—	7	33	46	7

XXI. Lymphocytopenia With Increased White Blood Count.

1. Relative in myelocytic leukemia.
2. Accompanying symptom in conditions with neutrophilia.
3. Severe infections.

XXII. Lymphocytopenia With Normal, Subnormal, or Very Low White Blood Counts.

- 1. Marked in measles shortly before and after the outbreak of the exanthem.
- 2. Sometimes in yellow fever and typhus.
- 3. Malignant attacks of certain diseases with lymphocytic changes (typhoid fever).
- 4. Paroxysmal hemoglobinuria during attacks.

XXIII. Monocytosis With Increased White Blood Count.

- 1. In health or slight illnesses if the first drop of blood from the ear is used without cleaning, and rubbing the ear.
- 2. Endocarditis ulcerosa (cells endothelioid and vacuolated).
- 3. Variola (with the "gay" blood picture).
- 4. Typhus in later stages.
- 5. Measles and German measles.
- 6. Varicella, less marked.
- 7. Slight in scarlet fever.
- 8. Less marked in parotitis epidemica, mild cases.
- 9. Less marked in acute syphilitic and tuberculosis processes.
- 10. Infections and infectious diseases as a secondary finding with good prognosis.
- 11. Monocytic leukemia.
- 12. Malignant endocarditis lenta ulcerosa (macrophages).

XXIV. Monocytosis With Low Normal to High Normal Counts of White Blood Cells.

- 1. Chronic vascular diseases, such as severe arteriosclerosis, in recovery, especially near the crisis.
- 2. Chronic syphilis and tuberculosis.
- 3. Slight in malignant tumors.
- 4. Acute protozoal diseases during the attacks (malaria, trypanosomiasis).
- 5. Severe cases of parotitis epidemica.
- 6. Basedow fruste.
- 7. Helminthiasis.
- 8. Hyporegenerative septic processes as a secondary finding.
- 9. Agranulocytosis.
- 10. Banti's symptom complex.
- 11. Measles.
- 12. Yellow fever.
- 13. Dengue fever.
- 14. Spotted fever.
- 15. Ophthalmia sympathica.
- 16. Poliomyelitis.
- 17. Pellagra.

Examples of Monocytosis and Leukocytoses

	LEUKOCYTES	BAS.	EOS.	MYEL.	JUV.	STAB	SEG.	LYMPH	MONO.	REMARKS
Variola—1st day	High	—	—	$\frac{1}{2}$	3	12 $\frac{1}{2}$	47	26	11	4 $\frac{1}{2}$ plasma cells
3rd day	Very high	—	—	—	1	2	29	45	23	5 plasma cells
4th day	High	—	—	—	1 $\frac{1}{2}$	6 $\frac{1}{2}$	12	46	34	5 plasma cells
8th day	Moderate	—	$\frac{1}{2}$	—	—	5	14	68	12 $\frac{1}{2}$	2 plasma cells
Typhus—10th day	—	—	—	—	—	6 $\frac{1}{2}$	34 $\frac{1}{2}$	20	16 $\frac{1}{2}$	7 plasma cells
Endocarditis ulcerosa	42,660	0.3	0.9	—	2	3.2	24.2	18.2	51.2*	
Tertian malaria	5,000	—	1	—	6	4	23	30	33	

*Of these monocytes, 35.3% were atypical, 6.1% were macrophages, and 9.9% were typical.

XXV. Decrease or Absence of Monocytes.

1. Very severe infections as a very unfavorable symptom.
2. Myelocytic and lymphocytic leukemias.

LEUKEMIA

TABLE 65.—LEUKEMIAS AND LYMPHOMAS*

TERM TO BE USED	TERMS TO BE AVOIDED
<i>Leukemia and Lymphoma</i> , generally, <i>Leukemia</i>	Leukosis, leukanemia, leukosarcoma, leukocythemia
Type unclassified	
Lymphocytic	Lymphoblastic leukemia or blast cell leukemia
Acute	Lymphadenosis, lymphocythemia, lymphatic, lymphoid or lymphogenous leukemia
Leukemic	
Subleukemic	
Aleukemic	
Subacute	
Leukemic	
Subleukemic	
Aleukemic	
Chronic	
Leukemic	
Subleukemic	
Aleukemic	
Granulocytic	Myeloblastic leukemia
Neutrophilic	Myeloid, myelogenous, myelocytic leukemia, mye- losis, myelemia, myelocythemia
Eosinophilic	
Basophilic	
Monocytic	Reticuloendotheliosis, histiomonocytic, Schilling type, myelomonocytic, or Naegeli type leukemia
Plasmocytic	
Megakaryocytic	
<i>Lymphoma</i>	Lymphoblastoma, lymphocytoma, malignant lym- phadenoma
Type unclassified	
Lymphocytic sarcoma	Lymphosarcoma
Reticulum cell sarcoma	Reticulosarcoma
Follicular lymphoma	Giant follicular lymphoma, giant follicular lympho- blastoma, lymphoid follicular reticulocytosis, Brill-Symmer disease
Hodgkin's disease, unspecified	Hodgkin's lymphoma, Hodgkin's lymphoblastoma, Hodgkin's pseudoleukemia lymphogranulomatosis
Hodgkin's paraganuloma	
Hodgkin's granuloma	
Hodgkin's sarcoma	
Mycosis fungoides	
Spiegler-Fendt sarcoid	
Plasmocytic myeloma	Multiple myeloma, plasma cell myeloma, plasmoc- ytoma, myelocytoma, Kahler's disease
Erythrocytic sarcoma	Erythroblastoma
Mixed types	

*Reproduced from the Am. J. Clin. Path. 20: 571-574, 1950, by courtesy of the Editor and of the Williams and Wilkins Company, Baltimore.

Leukemia is not a disease of a single species of cells in the blood or of a single hematopoietic organ. Rather it is a general disease of the systems that manufacture the leukocytes and also of the small cellular centers outside the ordinary blood-manufacturing centers. It is characterized by a more or less atypical hyperplastic new formation of white cells. These cases are eminently malignant and, therefore, are to be differentiated distinctly from simple metaplasia and from the leukocytoses. Leukemia is an extreme leukocytosis superimposed upon a specific irritation.

The historical development of leukemia might be said to begin with a description of the disease by Virchow in the eighteen fifties, when he outlined the "lienal" and "lymphatic" forms of leukemia. Between 1875 and 1877 Virchow differentiated leukemic cell increase from the leukocytoses due to pus formation. With the advent of selective stains into hematology there began recognition of the difference between the lymphocytic and the myelocytic leukemias. Then came Naegeli's characterization of the myeloblast which gave this dualistic conception of the disease a firm basis. With the final appearance of the oxydase reaction, it was possible to histologically differentiate myelocytic from lymphocytic cells.

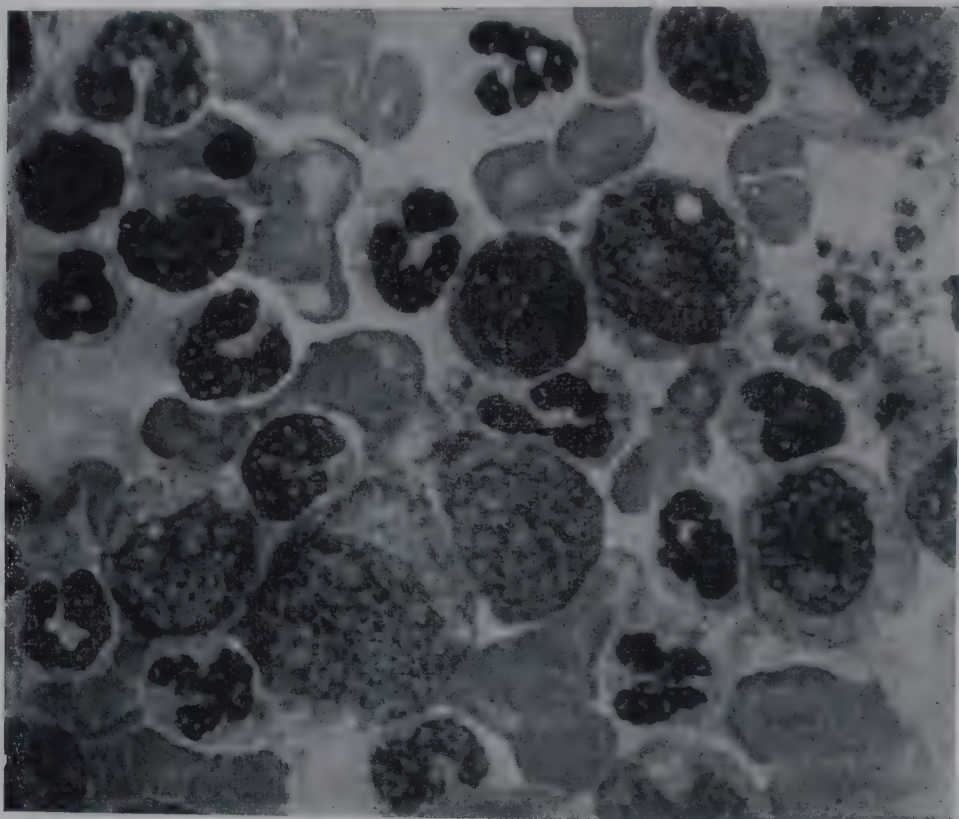


Fig. 199.—Myelocytic leukemia, blood film, showing crowding of cells, and an increase in large myelocytes and myeloblasts. ($\times 950$.)

The essential features of leukemias are seen in blood pictures. There is not only an increase in the total count of the leukocytes, but a qualitative cellular change as well. The disease is usually characterized by a prolonged incubation period, with general symptoms suggestive of climacteric or endocrine disturbances: lethargy, fatigue, rheumatoid pains—all symptoms that are not often taken to the physician for review. Frequently the palpable spleen or enlargement of glands is the first symptom noted by a physician of the existence of a leukemia. In advanced cases, there is pressure on the stomach, sometimes with vomiting, stinging pain in the splenic region, dyspnea, insomnia, perspiration, eventually subicteric color.

The etiology of leukemia is not known. Possible etiological causes are: (a) *Specific infection*. This has been often discussed but never proved. (b) *Generalized infectious irritation*. The anamnesis of a number of leukemia cases shows a series of infectious diseases, which suggests the possibility of an abnormal form of reaction to repeated infections. (c) There is a *tumor theory* due to the fact that there is a decided analogy to tumor processes in leukemia.

Ellermann has described a chicken-leukemia, which may be transferred by means of a filtrable virus, but which alternately shows myelocytic, lymphocytic, and erythropoietic hyperplasias. Snijders¹ reported in 1926 a large cellular leukemia of a guinea pig. One hundred forty-eight other guinea pigs which were inoculated with living cells from the first pig developed leukemia; some showed tumorlike infiltrations, some highly tumorous "leukosarcomatosis," etc. The proliferating cells all seemed to originate from the transplanted foreign cells. This may explain the development of generalized processes from a local tumorous tissue, and would also explain old descriptions of leukemias after crushing of the spleen, bone fracture, contusions, etc. (d) There is a theory of *disturbed correlation*. Ziegler is said to have experimentally produced genuine leukemias by destroying one blood system, followed by a degeneration of the other system. (e) There is a *constitutional* theory, but the literature on heredity in leukemias is entirely inadequate.

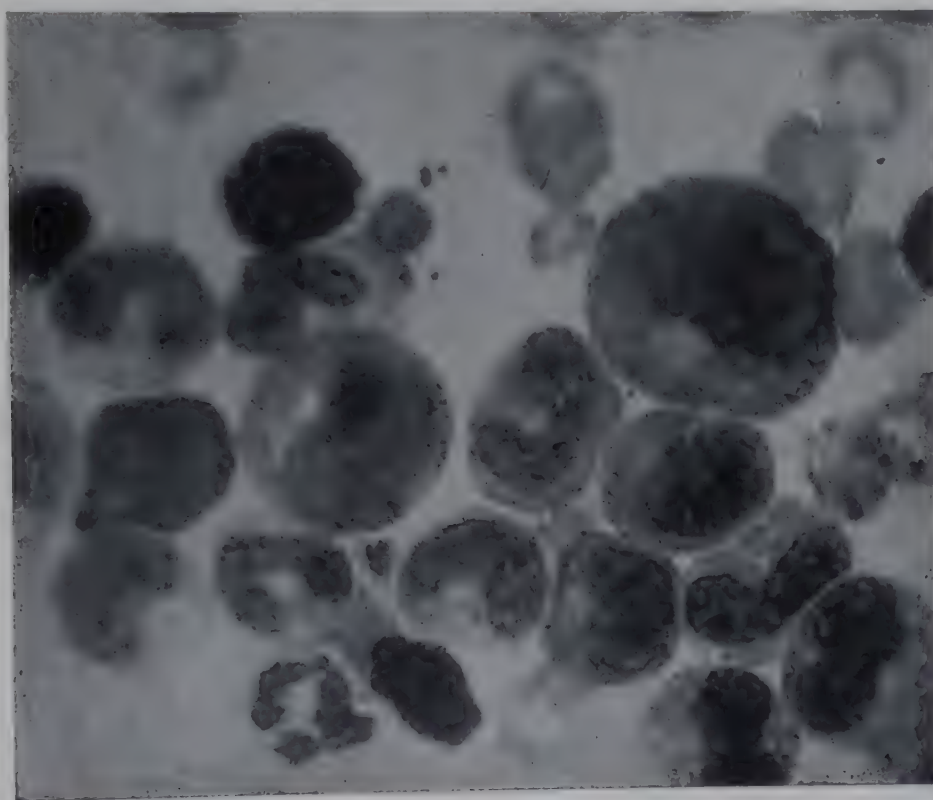


Fig. 200.—Blood film, leukopoesis, showing a large number of myelocytes and juveniles. (×950.)

Types of Leukemia

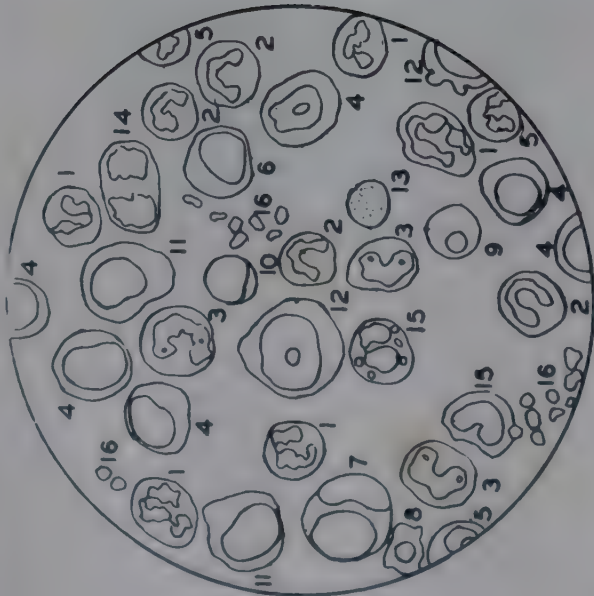
There are two main types of leukemia, acute and chronic. It is customary to classify the leukemias further on the basis of the predominating cell type into three classes: (1) myelocytic; (2) lymphocytic; and (3) monocytic. Other forms of leukemia have also been described: plasma cell leukemia, basophilic (?) leukemia, atypical leukemia, and leukemoid blood pictures.

The Chronic Leukemias

Chronic Myelocytic Leukemia.—Chronic myelocytic leukemia is characterized by highly active hyperplasia of the bone marrow which floods the blood stream with all the varieties of mature and immature leukocytes of marrow origin. In this disease there are a great many white cells, chiefly the immature

¹Snijders: Nederl. tijdschr. voor geneesk. 1926, 2nd Half, Vol. 11.J.

CHRONIC MYELOCYTIC LEUKEMIA



- 1. Segmented neutrophiles
- 2. "Stab"
- 3. Juveniles
- 4. Neutrophilic myelocytes
- 5. Eosinophiles
- 6. Eosinophilic myelocyte
- 7. Myeloblast with two nuclei without signs of mitosis
- 8. Polychromatic normoblast
- 9. Orthochromatic normoblast
- 10. Lymphocyte
- 11. Promyelocytes
- 12. Myeloblasts
- 13. Basophilic punctated erythrocyte
- 14. Erythroblast undergoing mitosis
- 15. Basophiles
- 16. Blood platelets

CHRONIC LYMPHOCYTIC LEUKEMIA



- 1. Lymphocytes
- 2. Immature lymphocyte
- 3. Eosinophile
- 4. Fragmentary nuclear structures
- 5. Basophilic stippled erythrocyte
- 6. Polychromatic erythrocytes
- 7. Normoblast
- 8. Large lymphocytes with azurophilic stippling
- 9. Segmented neutrophile
- 10. Monocyte
- 11. Blood platelets

PLATE XXII.

CHRONIC MYELOCYTIC LEUKEMIA

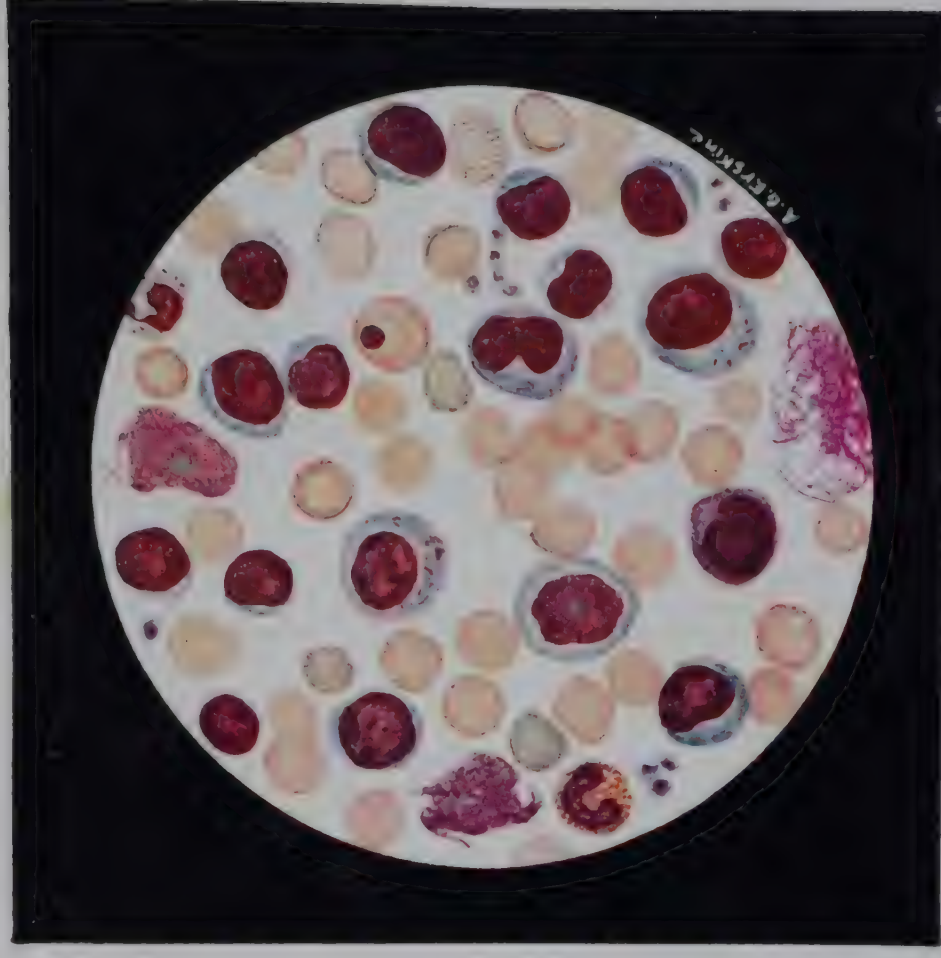
GIEMSA STAIN



X 950

CHRONIC LYMPHOCYTIC LEUKEMIA

GIEMSA STAIN



X 950



forms of the granular leukocytes, together with a progressive anemia showing many varieties of nucleated red cells. The name "leukemia" was given by Virchow to this disease because of the appearance of the blood at autopsy, namely, whitish, but this appearance is never seen during life.

The total white count is usually about 100,000 per cubic millimeter, but it may go as high as 1,000,000 per cubic millimeter. When the count is very high, it is necessary to use the red blood counting pipette in making the white count. (See page 577.)

The differential count of leukocytes varies in different patients. The neutrophile is the predominant cell in some cases, but this is not always true. Eosinophilic leukocytes are constantly increased in actual numbers and percentage. Basophiles may be increased up to 10 to 15 per cent of a high total count. Myelocytes are always present; usually they are numerous, sometimes forming one-half of the total white cells in the blood picture. The greater the blood count, the greater the number of myelocytes, especially in the later stages of the disease. Most of the myelocytes are neutrophilic, but there usually are eosinophilic and basophilic myelocytes as well. About 3 to 5 per cent of the cells in the differential count are myeloblasts with a few promyelocytes. There are usually less than 5 per cent of lymphocytes in the total count. There may be an occasional monocyte. The myelocytes are often atypical, varying in size and number of granules. Often the neutrophilic myelocytes retain the blue-staining cytoplasm which designates younger cells. There are many intermediate forms between the myelocyte and the "stabs" in this disease. These intermediate forms are called "juveniles" but many of them are atypical. With the oxydase stain, one can easily recognize just how many cells in the blood film are myeloid in origin.

The white cell count in the terminal stage may approach a normal figure due to the complete asphyxiation of the bone marrow function. In the terminal stage increasing immaturity in cell forms is seen. So many myeloblasts are seen that the picture suggests an acute myeloblastic leukemia.

It must be remembered that transitions from a chronic myelocytic blood picture to an acute myeloblastic one will occur and are connected with unfavorable changes in the course of the disease. A relapse or a terminal condition in chronic leukemia is usually attended with an acute blood picture.

Remissions are spontaneous. Apparent recoveries take place in leukemia. These remissions seem to date from the appearance of some intercurrent infection such as pneumonia, erysipelas, or pus infection; sometimes, tuberculosis. It seems likely that in the presence of a stimulus to leukocytosis the marrow reverts for a time to a normal and orderly leukopoiesis, returning later, of course, to its leukemic tendencies. These remissions sometimes last several months, but one must not be deluded into the thought during this period that the patient has been cured. Total count may drop from several hundred thousand to a normal figure following an acute infection, but sooner or later, the count insidiously begins to rise. Later on, with the end of the remission, the total count rises and the differential count becomes as characteristic as it was before. The red cell count, however, does not ever become normal during

a remission. Von Koranyi first used benzol in the treatment of leukemia with reduction of the white cells from 170,000 per cu.mm. to 8,000 per cu.mm. These apparently favorable results do not last long, so that the benzol treatment has fallen into disuse. Irradiation treatment of the long bones in the treatment of this disease is more popular today since it can be better controlled and produces less anemia. Refer to pages 888 ff. for the treatment of leukemia.

Red Cells and Hemoglobin in Leukemia.—Naegeli states that polycythemia is always present in mild cases of leukemia, but this is only for a very brief period. Anemia appears with hemoglobin falling to 60 per cent and red cells going down to 3,000,000. Color index remains around 1.0. As the disease progresses, the anemia becomes more severe. There is an increase in reticulocytes and many normoblasts appear in the picture. Typical megaloblasts are seen. Karyokinetic figures are found in the nuclei of these erythroblasts.

In the terminal stage of chronic myelocytic leukemia, the anemia becomes more marked as a result of which the patient usually dies. The picture at this point is very much like a true pernicious anemia.

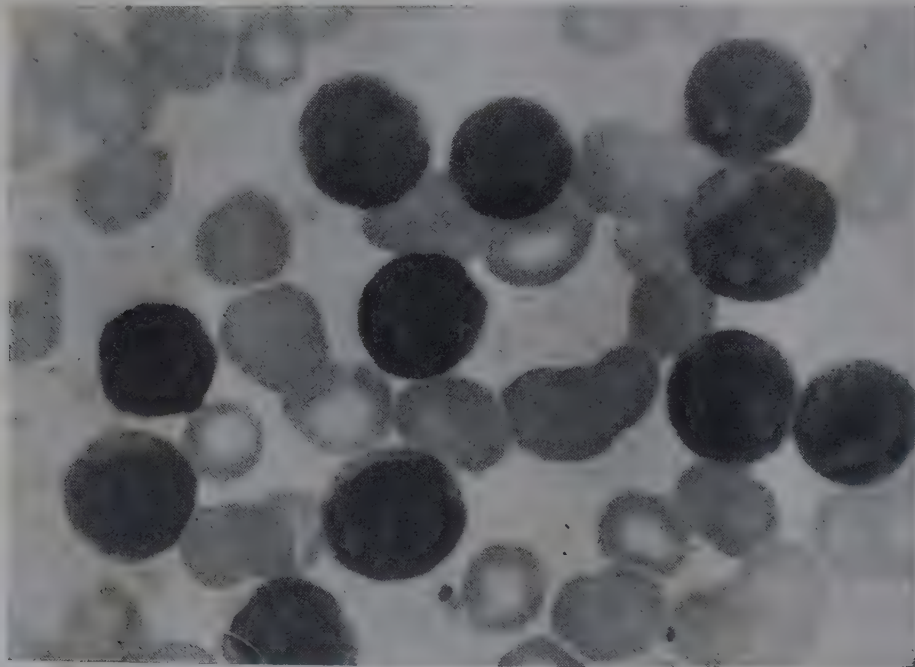


Fig. 201.—Lymphocytic leukemia, blood film, showing crowding of lymphocytes. ($\times 950$.)

Blood Platelets.—Blood platelets are increased to high figures. Minot and Buckman¹ reported as high as 2,000,000 blood platelets per cu.mm. The blood platelet count drops in the terminal stage.

Differential Diagnosis Between Chronic Myelocytic and Chronic Lymphocytic Leukemia.—The differential diagnosis between chronic myelocytic and chronic lymphocytic leukemia can be made on the basis of the generally larger spleen and by the absence of marked lymph node enlargement in chronic myelocytic leukemia. The *blood examination* is, of course, the best method of making the differential diagnosis. The differential count shows an increase in the neutrophilic elements in chronic myelocytic leukemia, whereas in lymphocytic leukemia, the increase is in the lymphocytes. The lymphocytes are relatively decreased in chronic myelocytic leukemia.

¹Minot, G. R., and Buckman, T. E.: *Am. J. M. Sc.* 169: 477, 1925.

Making a Differential Count of Blood in Myelocytic Leukemia

To make a differential count of blood in myelocytic leukemia, construct a recording sheet as shown on page 852 and proceed to count 500 leukocytes.

In reporting the count, divide the total results by 5 to convert to per cent, or the number of different types of cells in 100 leukocytes. Forms of reports vary in different institutions, but where no laboratory form is at hand, the following is suggested:

	M'blast	Pro.	B. Myel.	Baso.	Eo. Myel.	Eo. Juv.	Eo. St.
	10.2	6.2	0.6	1.4	2.2	0.4	0.6

Eo.	Neut. Myel.	Neut. Juv.	Neut. Stab.	Neut. Seg.	Lymph.	Monó.	Other Cells
3.8	16	12	8.6	36	0.8	1.0	0.2

For every 100 leukocytes counted there were:

0.8 P. megaloblasts, one with karyorrhesis

1.6 P. macroblasts, two with karyorrhesis

8.0 P. normoblasts, three with karyorrhesis

One erythroblast in mitosis found in the slide. Marked polychromasia, slight anisocytosis, enlarged blood platelets.

Chronic Lymphocytic Leukemia is a disease of middle and later life, rare under age 20 and most common after 50. The pathology is in the lymphatic tissues. The spleen is not usually as enlarged as in myelocytic leukemia. The spleen is engorged with lymphocytes and often shows areas of infarction. The bone marrow shows replacement of fat and replacement of normal red cell and myeloid cell tissues by lymphocytic infiltration. The lymph nodes of the entire body are enlarged, including not only the superficial glands of the neck, axillae, groins, etc., but the lymphoid follicles of the nasopharynx, tonsils, tongue, and gastrointestinal tract as well. The retroperitoneal and mediastinal nodes are often enlarged and cause symptoms by pressure. Cellular hyperplasia is shown throughout the body by the vast collections of large and small lymphocytes. There is an anemia present due to the crowding out of erythropoietic tissue by the lymphocytes. There is relative neutropenia.

The white count varies from 50,000 to 1,000,000 with an average of 100,000 per cu.mm. Eighty per cent or more of the cells in the differential count are lymphocytes of approximately the same size.* The remaining cells are neutrophils, including myelocytes, with a few eosinophiles, basophiles, or monocytes. Lymphoblasts and abnormal lymphocytic forms are seen in acute relapses. The red cell changes are those commonly seen in anemias connected with myelogenous or lymphatic dyscrasias (page 850). The blood platelets are reduced and there is a slight prolongation of bleeding time.

Lymphocytic leukemia is likely to show a transition of the blood picture to aleukemic state or even to acute state just as is seen in myelocytic leukemia. This occurs spontaneously or during the course of treatment or because of the onset of an intercurrent infection.

The Acute Leukemias

The Acute Leukemias.—(Myelocytic, Lymphocytic, Monocytic).—Friedreich¹ reported a case of leukemia with an acute course. Ebstein² in 1889

*The "monotonous" blood picture.

¹Friedreich, N.: Arch. f. path. Anat. 12: 37, 1857.

²Ebstein, W.: Arch. f. klin. Med. 44: 343, 1889.

described the clinical picture of acute leukemia. Fraenkel³ in 1895 called attention to changes in the cells in the blood and claimed that all acute leukemias were of lymphatic origin because of the similarity of the cells to lymphocytes. Naegeli⁴ in 1900 first described the myeloblast, furnishing the first data on which to classify two types of leukemia, myelocytic and lymphocytic. It remained for Schilling and Reschad⁵ in 1913 to describe their classical case of acute monocytic leukemia. This is considered the first case of this type reported, or the first case of reticuloendotheliosis, as some prefer to call it. Up to the time of Clough's report in 1932⁶ but twenty-three cases had been cited. In February, 1934, Forkner⁷ reported that during the past two years sixteen more cases of acute leukemia had been studied at the Thornlike Laboratory of the Boston City Hospital; these sixteen cases were divided into the three main types, myelocytic, lymphocytic, and monocytic (six of the latter). Post-mortem examinations were made in four of the six cases of monocytic leukemia. One case of myelocytic and one case of acute lymphocytic leukemia were included with autopsy findings.

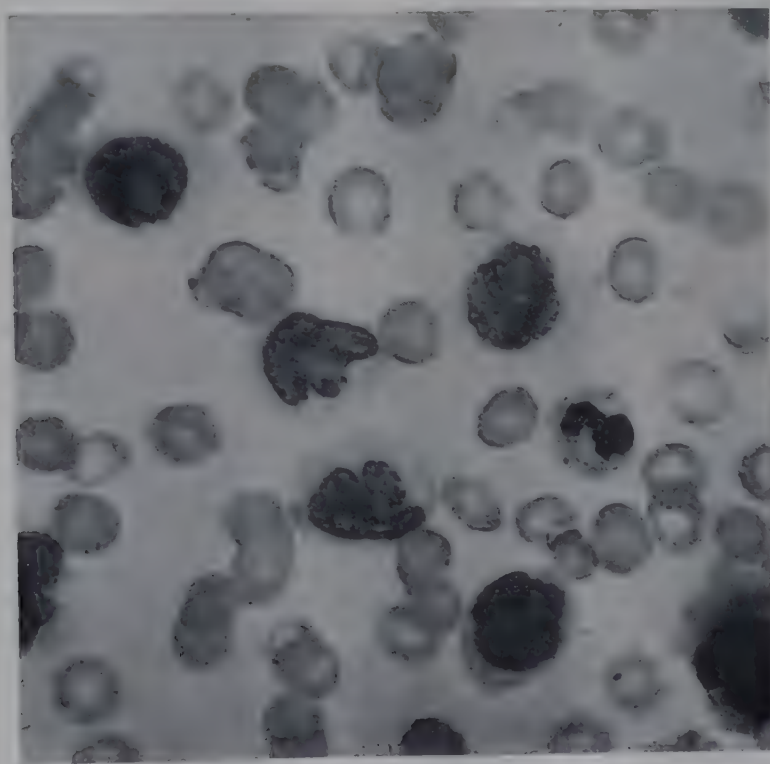


Fig. 202.—Monocytosis, blood film, showing large number of monocytes. (X950.)

Forkner called attention to certain clinical differences between acute monocytic and other forms of acute leukemia. He insisted that the clinical picture of diffuse, marked swelling of the mucous membranes, particularly of the gingivae usually with ulceration and necrosis, is characteristic of acute monocytic leukemia and is usually absent in acute leukemia of the other types. There was a diffuse cellulitis around the lesions of the mucosa, causing swelling and pain with signs of acute inflammation extending into the deeper tissues of the face. It was for this reason *that all these patients with monocytic*

³Fraenkel, A.: Deutsche med. Wchnschr. 21: 639, 663, 676, 699, and 712, 1895.

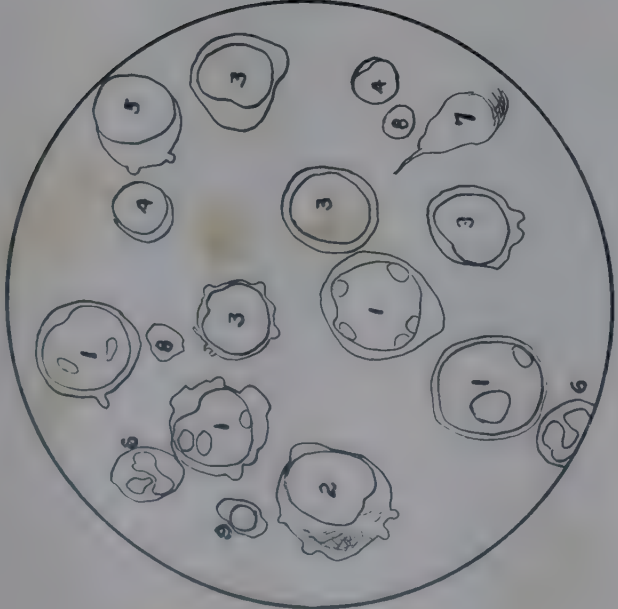
⁴Naegeli, O.: Deutsche med. Wchnschr. 26: 287, 1900.

⁵Schilling and Reschad: München. med. Wchnschr. 60: 1981, 1913.

⁶Clough, P. W.: Bull. Johns Hopkins Hosp. 51: 148, 1932.

⁷Forkner, Claude E.: Arch. Int. Med. 53: No. 1, 1934.

ACUTE LEUKEMIA



BLOOD FILM GIEMSA STAIN
×950

1. Lymphoblasts
 2. Prolymphocyte with Auer rods
 3. Atypical young lymphocytes
 4. Small lymphocytes
 5. Plasma cell
 6. Segmented neutrophile
 7. Fragmentary nuclear structure
 8. Polychromatic erythrocyte
 9. Normoblast
- Note lack of blood platelets

INFECTIOUS MONONUCLEOSIS



BLOOD FILM GIEMSA STAIN
×950

1. Atypical large lymphocyte
2. Plasma cell
3. Small lymphocyte
4. Neutrophilic "stab"
5. Segmented neutrophile
6. Blood platelets

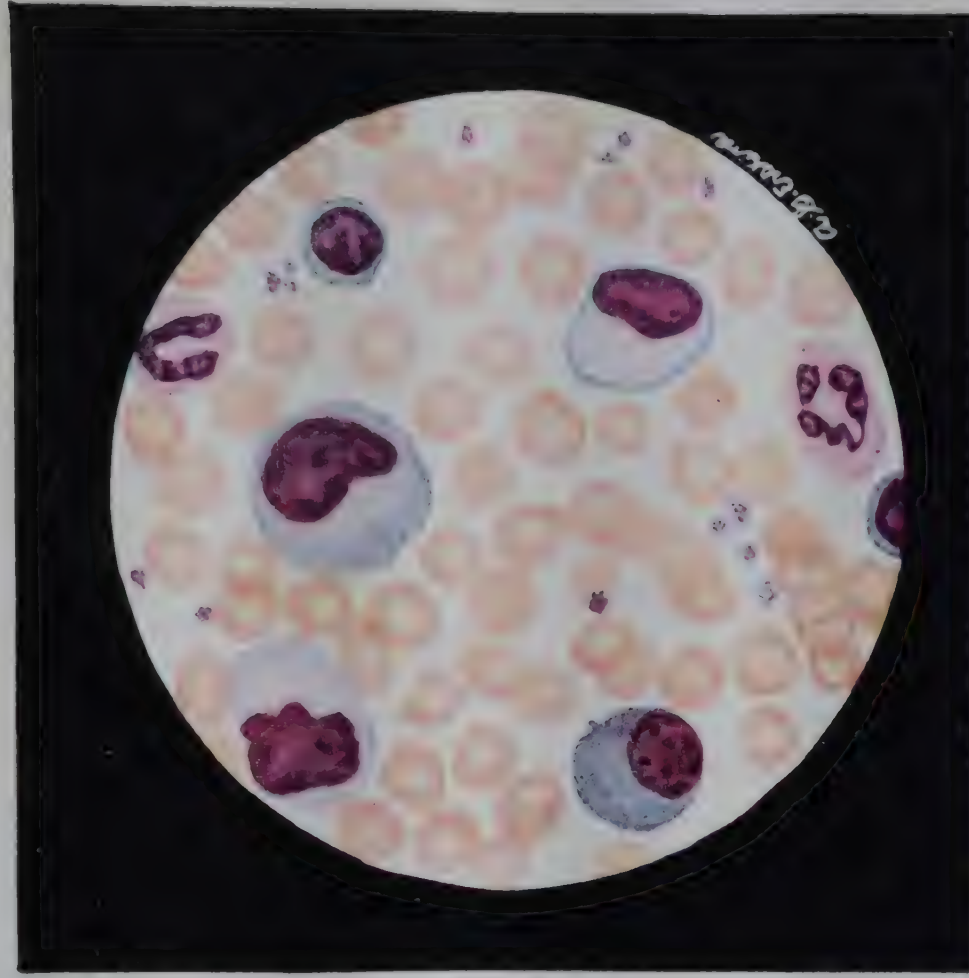
ACUTE LEUKEMIA



BLOOD FILM

GIEMSA STAIN X950

INFECTIOUS MONONUCLEOSIS



BLOOD FILM

GIEMSA STAIN X950

leukemia reported by Forkner consulted a dentist early in the course of their illness. While acute lymphocytic and myelocytic leukemia often have mouth lesions, they are different, consisting of hemorrhages and slight infection, not necrosis. This fact stands out prominently in the twenty-three cases reported by Clough⁸: eighteen had marked stomatitis or pharyngitis, abscesses of the alveoli, and four had swelling of the neck. Forkner studied the case histories of eight additional cases which were not accepted by Clough. Three cases reported by Ewald, Frehse and Hennig⁹ in 1922, one additional case by Bingel¹⁰ in 1916, one case by Dias¹¹ in 1930, one case by Hittmair¹² in 1930, and two cases by Asselstine¹³ in 1932 showed lesions of the mouth similar to those seen in the other eighteen cases tabulated by Clough.

There are other physical signs which serve to differentiate these cases. The spleen is somewhat enlarged but is not palpable in acute myelocytic leukemia but in the acute lymphocytic type it is palpable 2 to 3 cm. below the costal margin. The spleen in acute monocytic leukemia according to Clough was palpable in sixteen of the twenty-three cases recorded by him. In Forkner's six cases the spleen was palpable from 1 to 4 cm. below the costal margin. The weights of the spleens in the four cases studied by him at autopsy were: 1,065; 606; 360; and 900 gm., respectively.

The size of the lymph nodes should be used as an aid in the diagnosis of the type of acute leukemia. Forkner claims that there is invariably an enlargement of the lymph nodes in acute lymphocytic leukemia, but it is not so marked as in chronic lymphocytic leukemia. The lymph nodes, particularly those in the neck, may be slightly or moderately enlarged in acute monocytic leukemia, but we never see the enlargement in this disease that occurs in the acute lymphocytic type. There is only a negligible enlargement in acute myelocytic leukemia.

This clinical and histologic differentiation of the three types may be seen by studying Table 67, arranged by Forkner.

The characteristics above set forth will help the differential diagnosis, in the absence of a clear-cut blood picture or histologic study of the tissues at autopsy.

Blood Picture.—There has existed considerable confusion as to the cytology found in these three types of acute leukemia. This is enhanced by the fact that as many as 85 per cent of the leukocytes in any case of acute leukemia are of a uniformly immature type, usually in the myeloblast, lymphoblast, or monoblast stage of development. Intermediate stages are few in number. There are, too, some immature types present other than those of the typical leukemia with which we are dealing. Sabin,¹⁴ Simpson,¹⁵ and others, however, have rediscovered a method of aiding in the recognition of these cells; namely, the supravital method. One must not forget, too, that the oxydase test, as

⁸Clough, P. W.: Bull. Johns Hopkins Hosp. 51: 148, 1932.

⁹Ewald, Frehse, and Hennig: Deutsch. Arch. f. klin. Med. 138: 353, 1922.

¹⁰Bingel: Deutsche med. Wchnschr. 42: 1503, 1916.

¹¹Dias, A.: Monocytose algue: Leucemie monocyttaire, Rev. sud-am. de med. et de chir. 1: 41, 1930.

¹²Hittmair, A.: Folia haemat. 42: 271, 1930.

¹³Asselstine, S. M.: Folia haemat. 42: 271, 1930.

¹⁴Sabin, F. R.: Bull. Johns Hopkins Hosp. 34: 277, 1923.

¹⁵Simpson, M. E.: Univ. California Pub. on Anat. 1: 1, 1921.

TABLE 67.—CLINICAL AND HISTOLOGIC DIFFERENTIATION OF THE THREE MAIN TYPES OF ACUTE LEUKEMIA

	ACUTE MYELOCYTIC LEUKEMIA	ACUTE LYMPHOCYTIC LEUKEMIC	ACUTE MONOCYTIC LEUKEMIA
Spleen	Usually not palpable	Almost invariably significantly enlarged	Palpable in about 70 per cent of cases
Lymph nodes	Usually slight or no enlargement	General enlargement of moderate or marked degree	Moderate in neck, but other lymph nodes very slightly enlarged, if at all
Liver	Usually palpable	Usually palpable from 1 to 4 cm. below costal margin	Usually palpable from 1 to 4 cm. below costal margin
Mucous membranes, particularly of mouth and pharynx	Petechiae, bleeding; often slight swelling of gingivae but rarely ulceration	Petechiae, bleeding; rarely ulceration	Petechiae, bleeding; usually marked, diffuse swelling of gingivae or pharynx with ulceration; often cellulitis with swelling and tenderness of face; marked fetor oris
Oxydase reaction	From few to many oxydase-positive cells, with from few to many coarse granules	All lymphoid cells oxydase-negative	
Histologic distinctions of type cells in blood and tissues	Dominant cells myelocytes "A" (Sabin) and myeloblasts; nuclei round or oval, usually with several nucleoli; cytoplasm deeply basophilic; myelocytes "B" (Sabin) present; Auer's bodies frequently present in few cells	Dominant cells lymphoblasts and young lymphocytes; nuclei round, oval or slightly indented, with few nucleoli usually present; relatively small amount of deeply basophilic, hyaline cytoplasm; mature lymphocytes present in fair number; occasional myelocytes. "C" (Sabin) and metamyelocytes present	Dominant cells monoblasts and premonocytes; nuclei usually elongated and folded, deeply indented or otherwise irregular in contour; nucleoli rarely present; cytoplasm relatively abundant, usually not deeply basophilic and not hyaline but slightly basophilic and "ground glass-like"; Auer's bodies may be present in few cells; mature monocytes present in significant and varying numbers; myelocytes "C" and metamyelocytes often present in small numbers
Other characteristics			

used by Schilling and his followers, is of paramount importance in this regard. Forkner, with illustrated charts of cells found in cases, called attention to some striking points of identification in regard to myeloblasts; the nuclei are round, indented or oval, and contain several nucleoli. The cytoplasm is basophilic and of uniform character. The cells in monocytic leukemic cases are more mature, possess nuclei of complicated characteristics, with very few nucleoli; their cytoplasm is relatively abundant, is basophilic, and contains dustlike granules (see description of monocytes, page 641).

Histologic Differentiation of the Acute Leukemias.—The oxydase test for blood cells is a method for the differentiation of the tissues at autopsy in all leukemias. This is helpful criterion for the pathologist. Cells infiltrating the

liver, kidney, etc., if oxydase-positive, point to the myelocytic character of the case. If oxydase-negative, it is a case of lymphocytic leukemia or lymphoblastoma. In monocytic leukemia, the oxydase reaction may be positive or negative. Forkner calls attention to certain definite means of distinguishing monocytic cells in paraffin sections. Under the oil immersion lens, they show characteristics that will distinguish them from myeloblasts or lymphoblasts. The nuclei even of promonocytes and of some monoblasts are irregular in shape, in the form of an irregular crescent or a broad elongated nucleus bent upon itself. The nucleus is bent on itself several times, giving the impression of lobulation. This point made by Forkner is very impressive, especially since he has so insistently called attention to the "lobulation" of the nuclei of monocytes. May it not be true that this is not a lobulation but a pseudolobulation, as pointed out by Forkner? Distinct separation of the lobules is not demonstrable, in contrast to the ease of so doing with respect to the segmented neutrophile. There are also many young monocytic cells, with simple round, oval, or slightly indented nuclei, but these cells exist as close companions of the cells with the more complicated nuclei as described. Furthermore, there are other characteristics which serve to distinguish the monocytic cells from the early lymphoid and myeloid elements. The nuclei of the monocytic series of cells rarely have demonstrable nucleoli, whereas these structures are very common in lymphoblasts and myeloblasts. The cytoplasm of promonocytes and monoblasts is, as a rule, more abundant and much less basophilic than that of either lymphoblasts or myeloblasts and frequently it is so faint, with hematoxylin, that the border of the cell is barely recognizable. This is in contrast with the moderate or very deep basophilia of early lymphoid and myeloid cells.

In reference to the terms, acute and chronic lymphocytic leukemia, Wiseman³ has introduced the thought that from a hematologic point of view responses of the lymphatic tissue vary to such a degree that the terms acute and chronic are inadequate to express this change. Neither do these terms take into account the important relationship of lymphosarcoma to leukemia. "Finally," he states, "there does not appear to be any justification for the term 'aleukemic lymphatic leukemia,' since there is no fundamental pathologic difference between cases presenting low and high total white blood cell counts." He showed records that indicated that lymphocytic leukemia may be a very benign disorder which does not materially shorten life or measurably diminish health. On the other hand, it may kill quickly. It is probable that there are many more cases in the former group than one would suspect since many are asymptomatic, do not show physical signs, and hence are not seen by the physician.

Monocytic Leukemia (Leukemic Reticuloendotheliosis).—This is a rather rapidly fatal disease showing increased numbers of monocytes in the blood stream and in other organs. It was first reported by Reschad and Schilling⁴ in 1913. Rosenthal⁵ reported the first case in America in 1921. A large number of cases has since been reported, 127 in all in the American literature up

³Wiseman, B. K.: *J. A. M. A.* **107**: 2016, 1936.

⁴Reschad, H., and Schilling, V.: *München. med. Wchnschr.* **60**: 1981, 1913.

⁵Rosenthal, N.: *M. Clin. North America* **4**: 1607, 1921.

to June, 1937, and 45 cases of this disease are on file in the Registry of Blood Diseases of the American Society of Clinical Pathologists. Osgood⁸ reported six cases and Rosenthal and Harris and Doan and Wiseman found reports of 532 cases of leukemia, from which statistics on the incidence of monocytic leukemia were calculated. It was estimated that 62.2 per cent were cases of myelocytic leukemia, 32.7 per cent were cases of lymphocytic leukemia, and 5.0 per cent were cases of monocytic leukemia.

In considering this disease, the two schools of thought relating to the origin of blood cells must be taken into account; one school, of which Naegeli is a proponent, denying the reticuloendothelial source of the monocytic cells, and the other, of which Schilling is the leading proponent, insisting that these cells originate from reticuloendothelial structures. It can no longer be denied that there are certain cases of monocytic leukemia which start out to all intents and purposes as myelocytic leukemia. These cases inevitably become cases of pure monocytic leukemia. Naegeli has claimed that monocytic leukemia is a temporary monocytic phase of myelocytic leukemia, and that the monocyte is a descendant of the myeloblast. He denied that monocytic leukemia is a distinct pathologic entity and cited cases which at one time exhibited the hematologic features of monocytic leukemia and in which the typical blood picture of myelocytic leukemia developed later. From such cases he claimed to trace intermediate stages between myeloblasts and monocytes. Merklen and Wolf,⁹ Gittins and Hawksley, Reich, and others have reported cases supporting Naegeli's views. On the other hand, Schilling and his followers have maintained that monocytic leukemia is a distinct pathologic entity in which there is monocytosis in the circulating blood and hyperplasia of the reticular tissue in the hemopoietic organs. Schilling maintains that this disease is separate from myelocytic and lymphocytic leukemia. Forkner¹⁰⁻¹² believes that monocytes and their precursors are not a part of the reticuloendothelial system and that there is no clear-cut evidence of any intimate relationship between monocytes and the reticuloendothelial system, if the latter was considered in its strict sense. He likewise claims that any relationship of monocytes to myeloblasts has never been proved and he does not believe that the type of leukemia ever changed, for example, from monocytic to myelocytic leukemia.

It is for the reason that undoubted cases of myelocytic leukemia changing to monocytic leukemia have been reported by reliable workers that it is necessary to divide monocytic leukemic cases into two types: the first type, the monocytic leukemia of the Schilling order; and, second, the monocytic leukemia of the Naegeli type.¹³

Monocytic Leukemia of the Schilling Type.—Monocytic leukemia may occur at any age, the youngest patient reported being 11 months old and the oldest 78 years. The clinical course runs about the same as the other acute

⁸Osgood, E. E.: *Arch. Int. Med.* 59: 931, 1937.

⁹Merklen, P., and Wolf, M.: *Presse méd.* 35: 145, 1927.

¹⁰Forkner, C. E.: *Arch. Int. Med.* 58: 1, 1934.

¹¹Forkner, C. E.: *Leukemia and Allied Disorders*, New York, Macmillan Co., 1938, 333 pp.

¹²Forkner, C. E.: *A Symposium on the Blood*, University of Wisconsin Press, 1939, pp. 126-147.

¹³Downey, Hal: *Handbook of Hematology*, New York, Paul B. Hoeber, 1938, 2: 1275-1333.

leukemias, lasting from two weeks to a few months. Infection of the mouth or throat and spontaneous purpura are commonly seen. Swelling of the gums is a constant feature and is more marked and constant than in other cases of acute leukemia. This swelling is associated with gangrenous stomatitis. There is enlargement of the spleen and lymph nodes. Enlargement of the liver is also common, having been observed in 66 per cent of the 90 cases in which a notation was made. Other clinical symptoms are pallor, weakness, and fever. There are also cutaneous lesions of two types: nodules in the skin which are firm and painless, which on histologic examination prove to consist largely of monocytes, promonocytes, monoblasts, and reticular tissue; and staphylococcic infection of the skin, with multiple boils and carbuncles.

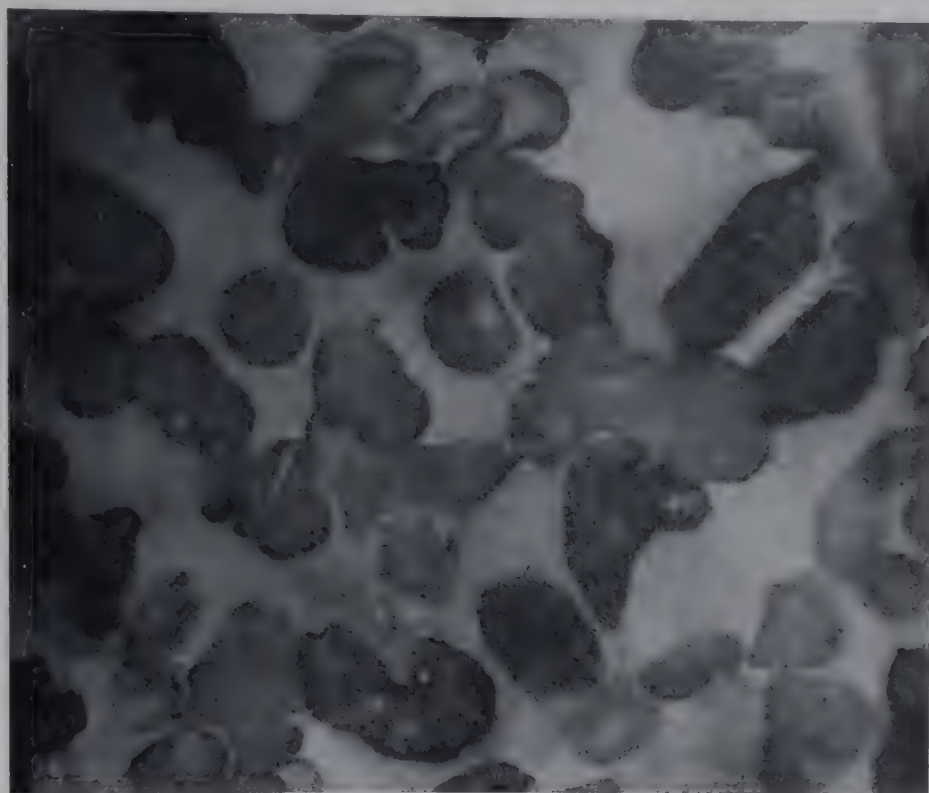


Fig. 203.—Endocarditis, showing endothelioid monocytes. ($\times 950$.)

The Blood Picture.—The blood picture shows a marked or moderate decrease in red cells and hemoglobin. There is a definite increase in the white cell count. The average of the highest counts recorded in each case was 99,600. A few cases showed leukopenia. Differential count showed mature monocytic cells forming about 50 to 75 per cent of the total count. Sometimes the immediate precursors of the monocyte, including the histiocytes, are present. The cytoplasm resembles that of the mature monocyte, being blue gray and of ground-glass appearance, with typical fine monocytic granules which are first seen about the nucleus in the youngest cells. The nucleus is large, oval, bean-shaped, or even lobulated. The chromatin is arranged in a very fine spongelike meshwork which becomes coarser and skeinlike in more mature cells. While most of the observers report that the immature cells are oxydase-negative, Farley¹ and Lawrence² and his co-workers found the cells to be oxydase-positive.

¹Farley, D. J.: *M. Clin. North America* 13: 991, 1930.

²Lawrence, J. S.: *Folia haemat.* 44: 332, 1931.

Histologically, the architecture of the lymph nodes and spleen is obliterated by a marked proliferation of reticular cells. Periportal growth is seen in the liver, and proliferation of reticular cells is found in the kidneys, the serosal surfaces, and the perivascular stroma of nearly all the organs, especially the adrenal glands, thymus, pancreas, and gastrointestinal tract.

The bone marrow shows a preponderance of monocytic cells. These are monoblasts which are very large, from 15 to 30 microns in diameter. Chromatin structure of the nucleus is very fine, similar to that of the myeloblast but even more transparent. The nucleus is usually round or oval. The cytoplasm ranges from basophilic to deeply basophilic, and there is more in proportion to the size of the nucleus than in the myeloblast or the lymphoblast.

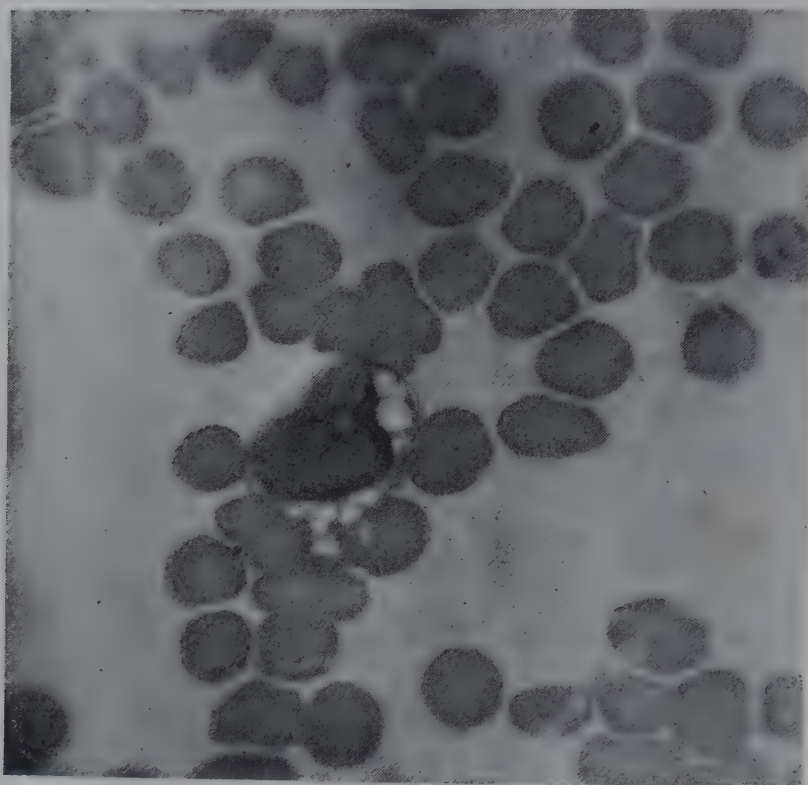


Fig. 204.—Blood film showing a phagocyte. ($\times 950$.)

As Osgood pointed out, monoblasts are difficult, sometimes impossible, to distinguish from myeloblasts or lymphoblasts. The chief difference is the more frequent occurrence of fine, diffuse azurophile granules in the cytoplasm of the monoblast, similar to those of the mature monocyte. Peroxidase stain does not assist in this differentiation, as all these cells may show a negative peroxidase reaction. Osgood, from his study of six cases and a review of the literature, definitely believes that the monocyte is developed from a cell which may be called the monoblast, similar in morphology to the myeloblast. This is very interesting in view of the fact that Forkner has listed nineteen different views as to the origin of the monocyte, which clearly shows the confusion that exists in regard to the origin of this cell. It is gratifying to know that a hematologist of Osgood's experience has so definitely accepted the entity of the monocyte as so determinedly maintained by Schilling and his followers all these years. Osgood has also commented upon the difficulty of distinguishing the blast cells from each other, namely, monoblasts, myeloblasts, lymphoblasts, and the plasmablasts. They are entities, of course, just as the megakaryoblast is, each of which can develop only into a mature cell of its own series. It

is to be hoped that cultures of marrow, which have been so well carried out by Osgood and Brownlee and Osgood and Muscovitz, will some day definitely settle this point. The type of cell which is found in the peripheral blood has been described by the various contributors to the literature of medicine on this question. Unfortunately, many of them have failed to differentiate between the mature and the immature cells of the monocytic variety in the circulating blood. Those who have been able to make this distinction have commented, of course, upon the finding in the peripheral blood not only of monocytes but of promonocytes, which are a more immature form of this cell.

Monocytic Leukemia of the Naegeli Type.—A report by Hall and Watkins¹ may be taken as an example of the monocytic leukemia of the Naegeli type, that is, beginning as myelocytic leukemia and changing to monocytic leukemia. This is similar to a case reported by Craciuneanu and Calalb² in 1931, which was as follows:

“The patient, a white woman aged 40 years, complained of fatigue, headache, insomnia, palpitation, and tumor in the left upper quadrant of the abdomen. These symptoms had developed three months previously. Later, she had experienced anorexia, vomiting, pallor, and weakness.

“Physical examination disclosed emaciation, pallor, a slight degree of adenopathy in the left cervical region, hepatomegaly, and a tremendously enlarged spleen descending 18 cm. below the left costal margin. Examination of the blood disclosed the typical picture of chronic myelogenous leukemia. Following a course of roentgen therapy for three weeks the leukocyte count dropped from 148,000 to 10,700 cells per cubic millimeter of blood. At the end of this time a differential count revealed a few cells that were called pathologic monocytes in addition to monocytes which appeared normal morphologically. These so-called pathologic monocytes were observed on two examinations of the blood during the ensuing month. Two and a half months after the first admission, examination of the blood revealed the monocytes to number 45 per cent of the total leukocytes. Many young forms, including cells called ‘monoblasts,’ were present. Following the one observation in which monocytosis was noted, the patient was not seen again. She died two months later; necropsy was not obtained.”³

The Hall and Watkins case was a woman, 23 years of age, admitted to The Mayo Clinic on June 4, 1935. Symptoms were failing strength, progressive loss of weight, fever, and pain in the left upper quadrant of the abdomen; these were of four years' duration. One year previous to admission, however, fatigue, generalized weakness, loss of weight, anorexia, and episodes of chilliness with fever, resulted in physical incapacity to carry on as a housewife. Extraction of a tooth was followed by hemorrhage. Diagnosis was made by her physician of myelocytic leukemia. There followed pain in the left upper abdominal quadrant, with nausea and vomiting. Physical examination showed fever, rapid pulse, weakness, emaciation, pallor, and appearance of critical illness. Superficial lymph nodes were enlarged. The spleen was enlarged, reaching to the level of the iliac crest. The total white count was 600,000 cells. The typical picture of chronic myelocytic leukemia was seen in spreads, with myeloid immaturity to myeloblasts, the predominance of young forms being in the neutrophilic series. There was severe secondary anemia. After treatment, patient went home but after two months was returned to the Clinic. One month after her first visit the white count had dropped to 100,000; three weeks later it had risen to 400,000. X-ray therapy was advised. Examination at this time showed slight anemia and a white count of 60,000, with less myeloid immaturity in the blood spreads, the youngest forms found being neutrophilic promyelocytes; there were no myeloblasts or leukoblasts; 5 per cent of the leukocytes were monocytes. No monocytes were seen in the spreads at the time

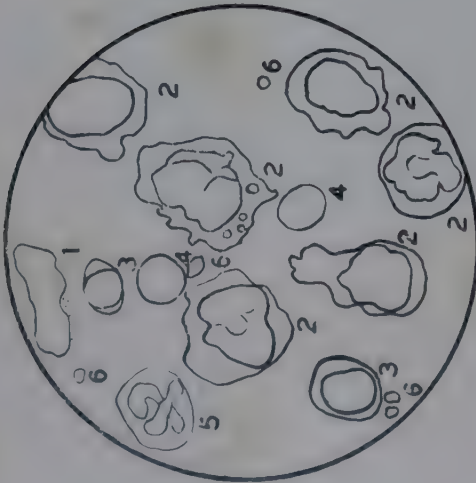
¹Hall, Byron E., and Watkins, Charles H.: *Am. J. Clin. Path.* 11: 443, 1941.

²Craciuneanu, A., and Calalb, G.: *Sang.* 5: 397, 1931.

³Quoted from Hall & Watkins, loc. cit.

Monocytic Leukemia

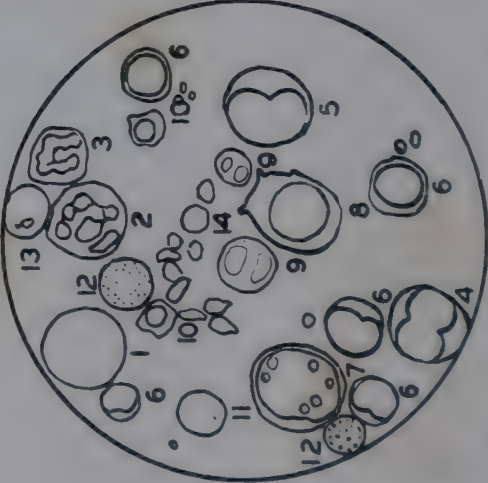
- 1. Endothelioid monocyte
- 2. Monocytes
- 3. Lymphocytes
- 4. Polychromatic erythrocytes
- 5. Segmented neutrophile
- 6. Blood platelets



MONOCYTIC LEUKEMIA

Von Jaksch's Disease

- 1. Megalocyte
- 2. Segmented neutrophile
- 3. Neutrophilic "stab"
- 4. Neutrophilic juvenile
- 5. Neutrophilic myelocyte
- 6. Lymphocytes
- 7. Lymphoblast or myeloblast
- 8. Plasma cell
- 9. Polychromatic erythrocyte with endoglobular degeneration
- 10. Polychromatic microblasts
- 11. Polychromatic erythrocyte
- 12. Erythrocytes with basophilic punctation
- 13. Blood platelet on a red cell
- 14. Blood platelets
- Marked anisocytosis



VON JAKSCH'S DISEASE
PLATE XXIV.

Trichinosis

- 1. Monocyte
- 2. Neutrophilic "stab"
- 3. Eosinophiles
- 4. Crushed eosinophile
- 5. Blood platelets



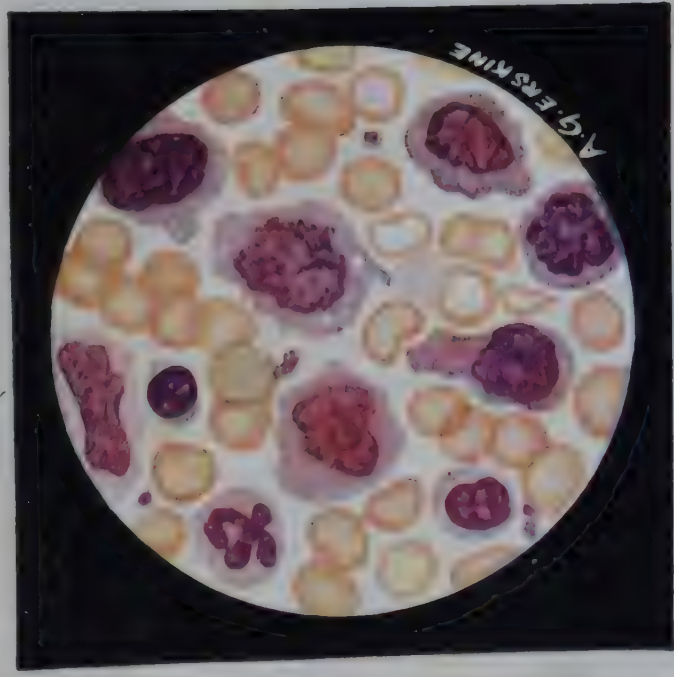
TRICHINOSIS

Chlorosis

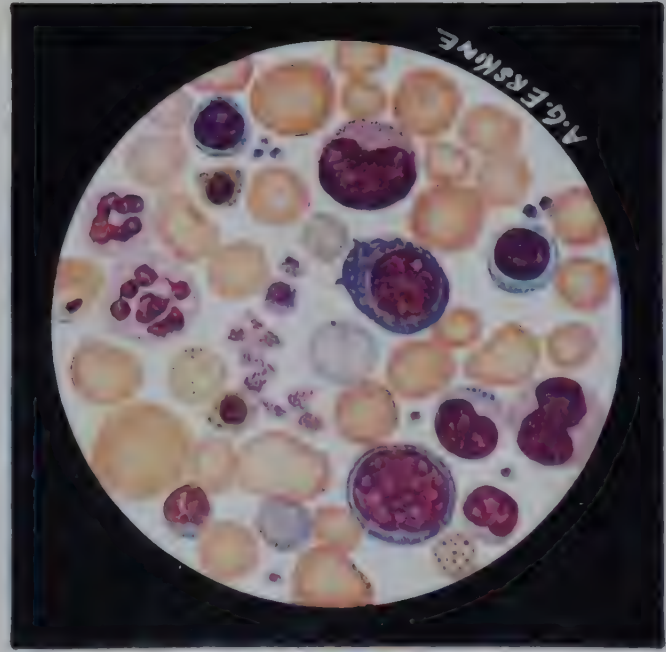
- 1. Segmented neutrophiles
- 2. Polychromatic erythrocytes
- 3. Poikilocyte with endoglobular degeneration
- 4. Poikilocytes
- Hypochromic microcytic anemia
- Marked poikilocytosis and anisocytosis



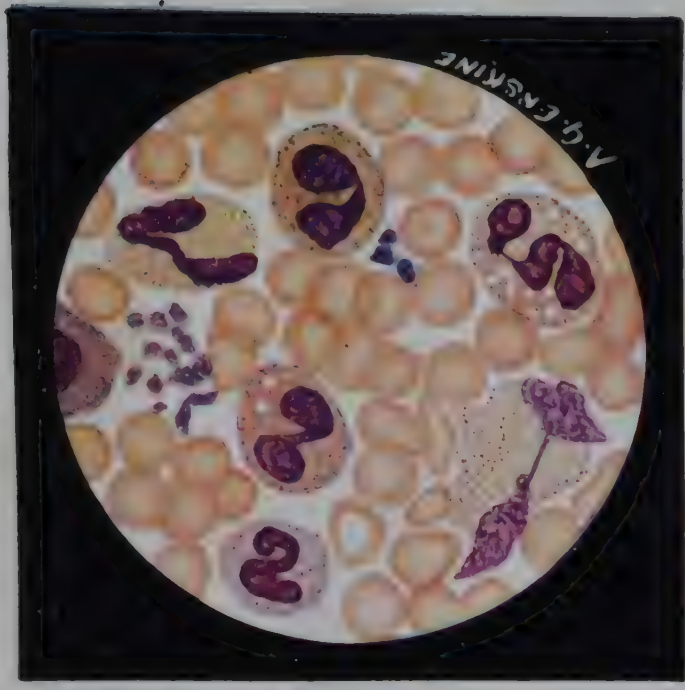
CHLOROSIS



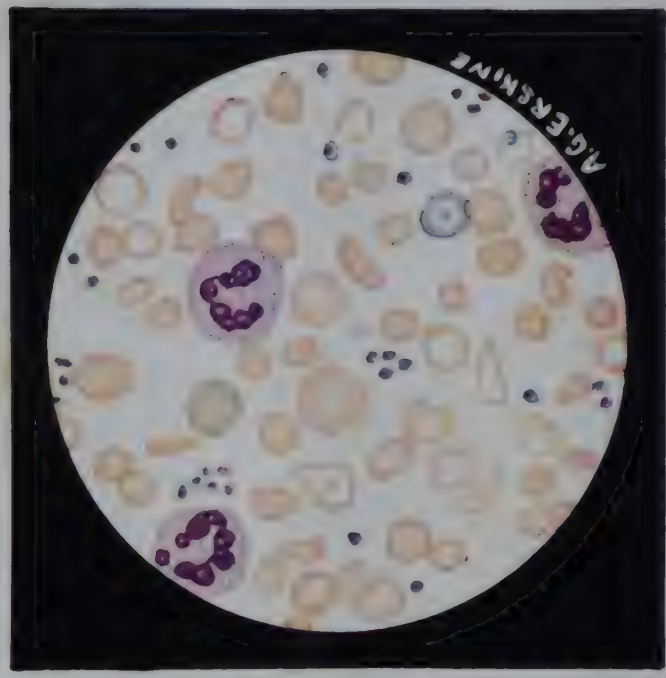
MYELOCYTIC LEUKEMIA



VON JAKSCH'S DISEASE



TRICHINOSIS



CHLOROSIS

of the first admission. Roentgen treatments were administered daily. After seven days, she was dismissed but returned after three months. The spleen had receded in size, and the leukocyte count was 13,400. There was no anemia. The blood films now showed the predominant cell to be the monocyte. At this time she had 51.5 per cent monocytes and 12.0 young monocytes. Immature granulocytes were infrequent. She was discharged but readmitted on Dec. 25, 1935, because of marked dyspnea, vomiting, and abdominal pain. Diagnosis at home had been pneumonia. Spleen had enlarged. At this time, she showed 58.0 monocytes, of which 23.5 per cent were young monocytes. She was taken home against the advice of attending physicians. At the time of dismissal, leukocytes were 36,000 cells per cubic millimeter, with blood picture the same as at the time of admission. She rallied but died, after two months, on March 7, 1936. Blood films showed at this time 59.0 per cent monocytes, of which 34.5 were young monocytes. No necropsy was obtained.

In this case there can be no doubt that there was a conversion in the blood picture from typical myelocytic leukemia to one that was clearly monocytic leukemia, and that after the monocytic leukemia had developed the monocytes could be traced back to stem cells. No cells typical of reticuloendothelial cells were seen in the circulating blood of this patient at any time. Therefore, Hall and Watkins believe that the monocyte was derived from a cell which possessed all the characteristics of the myeloblast. The number of stem cells seen in the blood after the picture of monocytic leukemia had developed was small. Hall and Watkins believe that a "monocytic phase" may develop at any time in the course of myelocytic leukemia or that the picture may be that of monocytic leukemia when the patient is first seen, later changing to one of chronic myelocytic leukemia which at a later date changes to one of monocytic leukemia as was demonstrated by the case above reported. Levine¹ reported a case which was studied for a long time because of severe anemia of indeterminate type. Ten days before death, a leukemic blood picture developed in which myeloid immaturity toward the stem cell, and mature and young monocytes were observed, with the findings at necropsy of subacute myelocytic leukemia. Levine expressed the opinion that this picture illustrated the development of a monocytic phase in the course of myelocytic leukemia after an initial aleukemic phase.

Hall and Watkins studied all of the cases of monocytic leukemia seen at the Mayo Clinic in a ten-year period and found that twenty-three cases were of the Naegeli type and six were of the Schilling type. They believe that the Naegeli type of monocytic leukemia should be considered a variant of myelocytic leukemia and the Schilling type a variant of leukemic reticuloendotheliosis.

The blood picture given below represents a typical case of monocytic leukemia of the Schilling type. The case record is from our service at the Christian Hospital, St. Louis, Mo., and was obtained through the courtesy of Dr. D. L. Twedell.

Patient was a 36-year-old white male, married, the father of one small child. Patient had been ill since March 21, 1942. Symptoms began with severe abdominal pain, general lymphadenopathy, especially of the groin and neck; fever, weakness, headaches, general malaise. Patient lost much weight. Condition became steadily worse, and patient entered a hospital. Two weeks later he transferred to the Christian Hospital. General condition: greatly emaciated, anemic looking, poor condition. Mentally very depressed, seeming to live in fear of dying. Liver and spleen enlarged, palpable, and tender. Heart sounds of fairly good quality. Bronchovesicular breathing showed no definite dullness. Moist râles

¹Levine, V.: *Folia haemat.* 52: 305, 1934.

over the bases. Kidneys not palpable. Patient had difficulty in urinating. Reflexes deep and superficial, weak.

On admission the pulse was 110; temperature, 101° F.; respiration, 28. Temperature gradually increased from 103 to 105°. The patient received 500 c.c. of citrated blood on the second, third, and sixth day of hospitalization. Patient expired one week after entrance.

Blood pictures follow:

HOS- PITAL DAY	W.B.C.	R.B.C.	HB. SAHLI	C.I.	E.	B.	M.	J.	ST.	SEG.	L.	MONO.	OTHER FINDINGS
1st	105,000	1,200,000	20%	0.8	1	-	-	-	-	9	5	85	5 p. normo- blasts 4 o. normo- blasts
6th	360,000	1,770,000	35%	1.0	-	-	-	-	-	4	19	77	1 p. normo- blast 3 o. normo- blasts

There have been a number of erroneous conclusions about monocytic leukemia due to the fact that only single observations have been made on some cases. Thus, at various times, the features of agranulocytosis, aleukemic myeloid leukemia, aplastic anemia, leukemoid monocytosis, leukopenic infectious monocytosis, chronic splenic panhematopenia, and reticuloendotheliosis have been depicted. Monocytic leukemia may pursue a very erratic course. This was exemplified in the unusual case reported by Rappoport and Kugel¹ This case was exhaustively studied. There were four sternal punctures. There were several episodes of granulocytopenia. Serial blood counts were made and autopsy findings were very complete in this case, and the conclusion was drawn that this was unquestionably a case of true monocytic leukemia. The authors believe that the distinction between the Schilling and Naegeli types of monocytic leukemia is more apparent than real and does not deserve the rigid segregation frequently employed and heretofore alluded to in this chapter. A similar contention has been advanced by Campbell, Henderson, and Croom² and Custer.³ This case illustrated, as do most of the cases, that in the later stages of the disease, as the frank monocytic leukemia progresses, the myeloid reaction diminishes. This myeloid reaction is, for the most part, an expression of the bone marrow reactivity to the agranulocytosis and is not an expression of the monocytic leukemia.

There are certain essential facts which must be borne in mind relative to the diagnosis of this disease. One of these facts is the proper identification of the monocyte. It is usually larger than the granulocyte; its cytoplasm is opaque, mottled, gray-blue, and contains azurophilic granules. The cell margins are serrated and irregular. The nucleus is reniform or horseshoe-shaped and possesses a lacy, skeinlike, coarse chromatin weave. With supravital stain, numerous neutral red or salmon-pink colored bodies, vacuoles, are symmetrically arranged around a centrosphere located in the bay of the nucleus. This structure is the rosette which is considered so typical of the mature monocyte. Moreover, the monoblast measures from 15 to 20 μ in diameter and in fixed stained

¹Rappoport, A. E. and Kugel, V. H.: Blood, Vol. II, No. 4, 332-355, July, 1947.
²Campbell, A. C. P., Henderson, J. L., and Croom, J. H.: J. Path. & Bact. 42: 617, 1936.
³Quoted by Rappoport and Kugel from a personal communication.

films it possesses a basophilic cytoplasm containing no granules and, distinctively, no Auer bodies. The nucleus is round, large, and centrally situated; its chromatin is fine, stippled, and sievelike, and usually possesses two nucleoli.

Rappoport and Kugel emphasize that this diagnosis requires prolonged and intensive clinical and laboratory observations. The differential diagnosis depends solely upon the results of bone marrow examinations. The presence of "reactive" or "irritative" or the leukemoid phenomena or intoxications must be considered and evaluated in the differential diagnosis.

Lymphocytic Leukemia Following Benzol Poisoning.—Falconer¹ reported a case of chronic benzol poisoning, gradually developing the picture of a typical leukemia as follows:

This man, aged 58 years, was employed in a can factory. The can ends, rimmed with a solution of rubber in benzol, were delivered to him by a conveyor, the benzol evaporating from the rubber mixture en route. Falconer had seen four cases of typical benzol poisoning from the same plant during the previous year. This patient's blood studies showed 50% hemoglobin, 1,530,000 red blood cells, 8,100 white blood cells, 62% neutrophils, 32% lymphocytes and 6% monocytes. Blood platelets numbered 68,000; red cells showed anisocytosis and some polychromasia. The symptoms were diagnosed as chronic benzol poisoning. Later on, hemorrhages from the nose began, together with decrease in red and white counts. After a blood transfusion, the nasal bleeding ceased. About a month later he returned for treatment, and two months later the bleeding from the nose reappeared. The blood picture showed decrease in the number of red cells and amount of hemoglobin, but white cell volume rose. About four months later, he showed a picture of beginning lymphocytic leukemia, the counts running up to 50,000. This state persisted for about a year when death occurred, with a clinical diagnosis of lymphocytic leukemia.

There were three important questions suggested by a study of the clinical data and autopsy material on this patient. The autopsy material, by the way, showed general lymphadenopathy, increase in size of spleen, leukemic infiltration of liver and kidneys, secondary anemia, bronchopneumonia, and purulent bronchitis. The lymph nodes showed a striking overgrowth of tissue by a type of cell which was larger than the usual lymphocyte and which contained a thin rim of cytoplasm. The bone marrow showed the same type of cell as the lymph node.

The questions are:

1. What effect does benzol have on lymphatic tissue?
2. Is it not possible for benzol, like other well-known hemopoietic irritants, to upset the hemopoietic balance between cell growth and destruction, giving rise to lymphoid hyperplasia?
3. Does this lymphoid hyperplasia always stop just short of leukemia, or can it, under prolonged irritation or in susceptible individuals, progress to lymphocytic leukemia?

Lignac² injected mice with from 25 to 30 injections of benzol (0.1 c.c. of 0.3 c.c. benzol in 10 c.c. olive oil) and concluded that the symptoms of hyperplasia developed and that the spleen, which was first hypertrophied, decreased in size. The animals showed hyperplasia but no lymphocytic leukemia. He did not succeed in producing lymphocytic leukemia. The hyperplasia he con-

¹Falconer, E. H.: *Am. J. M. Sc.* 186: 353, 1933.

²Lignac, G. O. E.: *Krankheitsforschung* 6: 97, 1928.

sidered as a tumorous growth similar to leukemia. It is a real tumor of precursors of white blood cells. He stated that the immaturity of undifferentiated cells suggests acute leukemia, and the development of tumor may, or may not, be accompanied by a leukemic blood picture.

Thermal and Chemical Influences on Hematopoiesis.—

Murphy and Sturm² reported experimental work with hemopoietic irritants, accomplished by subjecting mice, rats, and guinea pigs to dry heat at temperatures of from 55° to 65° C. with short exposures of about 5 minutes; white counts were made at intervals over a 3-week period. The blood picture showed an immediate drop in both neutrophils and lymphocytes, which averaged about 3,000 cells per cu. mm. Seven days after the heating, there was an increase of from 5,000 to 10,000 cells per cu. mm., the increase being almost entirely in the lymphocytes. Fourteen days after heating, the lymphocytes had increased to from 12,000 to 14,000 per cu. mm. above the original counts; the neutrophils remained low and did not increase until about three weeks after heating. From 6 to 10 days after heating, several lymphocytes were noted apparently in process of amitotic division. These cells in the process of division looked very much like the cells reported by Falconer in his case of leukemia following benzol poisoning.

Nakahara,³ in trying to determine the source of lymphocytosis induced by heat, found the first change in the spleen and the lymph glands with widespread degeneration of the lymphoid elements. This was followed in 48 hours by activity of the "germinal centers" with an overproduction of lymphocytes, the spleen and lymph glands becoming larger than normal. There was a marked increase of mitotic figures in the lymphocytes of the germinal centers following the general necrosis.

Benzol presumably may act on lymphatic tissue in a manner similar to dry heat, causing destruction first then a compensatory hyperplasia, exceeding the normal limits. It is questionable whether this might not later result in leukemia.

The influence of chemicals on lymphatic tissue has been studied. Isaacs⁴ found that arsenic acts as a depressor of the bone marrow with decreased production of young red blood cells. If arsenic is stopped, the bone marrow responds once more with an increase in the rate of maturation of the erythroblastic tissue and an increased production of young red blood cells. Balbridge, Rohner, and Hausmann⁵ found six cases of infectious mononucleosis which developed under antisyphilitic treatment. White⁶ reported a case of infectious mononucleosis which also developed under antisyphilitic treatment. Falconer in 1925 (unreported) observed a case with infectious mononucleosis with a severe Vincent's infection of the buccal cavity after neoarsphenamine administration in syphilis.

²Murphy, J. B., and Sturm, E.: *J. Exper. Med.* 1: 29, 1929.

³Nakahara, W. J.: *J. Exper. Med.* 29: 17, 1919.

⁴Isaacs, R.: *Folia Haemat.* 37: 389, 1928.

⁵Balbridge, C. W., Rohner, F. J., and Hausmann, G. H.: *Arch. Int. Med.* 38: 413, 1929.

⁶White, E. C.: *U. S. Naval Med. Bull.* 22: 302, 1928.

Farley¹ and others believe that the benzol ring in arsphenamine and its derivatives may be responsible for hemopoietic tissue damage. Others, such as Sternberg,² suggest that acute leukemia is an infection. Cabot³ reported leukemoid blood pictures following lymphangitis, furunculosis, acute streptococcus tonsillitis, and cervical adenitis. Herz⁴ mentioned cases of infection with a blood picture simulating lymphocytic leukemia. Marchand⁵ reported a case of severe sepsis with bone marrow exhaustion and a picture of lymphoid hyperplasia.

This report does not prove that benzol may cause lymphocytic leukemia. It does stimulate regeneration of the erythropoietic and leukopoietic tissue, chiefly in the bone marrow. In an individual with a labile lymphatic apparatus as Farley's case, the lymphatic response may be unusual and carry on to a leukemic state. The individual was a short, obese patient with hypertrophied tonsillar tissue, who might be termed a "lymphatic" type.

The effect of radium and x-rays on hemopoietic tissue has not yet been worked out. Cases of leukemia and of aplastic anemia in workers exposed to x-ray and radium have been reported. Emile-Weil⁶ reported two cases in engineers working in the same laboratory with radioactive material. One died with pernicious anemia and the other with myelocytic leukemia. Farley⁷ states, "In view of the undoubted production of aplastic anemia by the action of rays on the bone-marrow, it is not surprising that the production of a myelogenous type of leukemia by rays should be reported, but the production of lymphatic leukemia by such means should not be credited without very close scrutiny."

Experimental work carried on at the Rockefeller Institute⁸ shows that with a certain dose of the roentgen ray, a lymphocytosis similar to that produced by heat can be brought about, that is, first a lymphocyte fall, followed by a marked rise.

Studies^{9, 10} on people exposed to atomic bomb radiation in Hiroshima and Nagasaki revealed 75 cases of leukemia, indicating a great increase in incidence of this disease in the survivors of this exposure. The predominant form was chronic myelocytic leukemia, but it has been pointed out that lymphocytic leukemia is rare in Japan. The leukemogenic effect of ionizing radiation was distributed equally in all age groups and in both sexes. In some of the cases there was a prolonged latent period of several years before development of the disease. In these preclinical stages, disturbances of the red cells and platelets as well as of the leukocytes were noted. An increase in basophiles, many of which were abnormal, was one of the outstanding features. These authors

¹Farley, D. L.: *Am. J. M. Sc.* **179**: 214, 1930.

²Sternberg, C.: *Verhandl. d. deutsch. path. Gesellsch.* **16**: 81, 1918.

³Cabot, R. C.: *Am. J. M. Sc.* **145**: 335, 1913.

⁴Herz, A.: *Die akute Leukämie*, Leipzig, 1911, Franz Deutike.

⁵Marchand, F.: *Deutsch. Arch. f. klin. Med.* **110**: 359, 1915.

⁶Emile-Weil, P.: *Presse méd.* **33**: 1297, 1925.

⁷Farley, D. L.: *Medicine* **7**: 65, 1928.

⁸Murphy, J. B., and Morton, J. J.: *J. Exper. Med.* **22**: 800, 1915; Thomas, M. M., Taylor, H. D., and Witherbee, W. D.: *J. Exper. Med.* **29**: 75, 1919.

⁹Lange, R. D., Moloney, W. C., and Yamawaki, T.: *Blood* **9**: 574, 1954.

¹⁰Moloney, W. C., and Lange, R. D.: *Blood* **9**: 663, 1954.

hypothesize that disturbances in cell enzyme systems with a resultant loss or alteration of cell growth-promoting or regulating factors account for the leukemic changes.

Basophilic (?) Leukemia

There is some dispute as to whether or not there is actually an entity which may properly be called basophilic leukemia. Many contend that these are cases of chronic myelocytic leukemia with large numbers of basophiles. Groat and others¹¹ reported a case of acute basophilic leukemia which seems to bear out their belief that this entity may actually occur. They cite the report of Joachim,¹² who in 1906 reported two cases of chronic leukemia in both of which the percentage of mast cells or basophilic cells was above 50. The blood picture in Joachim's case was of an ordinary chronic myelocytic leukemia except for the large number of mast cells, as high as 83%. Joachim's opinion was that the general symptoms were similar to other chronic myelocytic leukemias except for the degree of basophile increase. He reported it as a case of atypical leukemia, concluding that chronic basophilic leukemia is a variety of the common myelocytic variety. Sabrazès¹³ in reporting a case of chronic myelocytic leukemia with many mast cells stated that the so-called hemohistioblast of Ferrata and many types of basophiles were frequently found increased in chronic myelocytic leukemia. Elliott and Young¹⁴ reported a case of chronic mast-cell leukemia because of predominance of leukocytes with coarse basophilic granulation.

The case reported by Groat and others was a young man, 27 years old, with a blood count of 186,000 white blood cells on first observation, but no basophilic element was originally present. Having been treated with roentgen ray applied to the splenic area, he returned some months later with a total count of 10,200 white cells and 2% basophiles. About two months later, his blood showed 12% basophiles. He died about two months after the above observation after repeated x-ray treatments. Post-mortem bone marrow examinations showed eosinophilic myelocytes in large numbers and many myelocytes, which were probably divided between the basophilic and neutrophilic types. Megakaryocytes were present in small numbers. Groat concluded that they would like to call their case acute myeloblastic leukemia with extreme basophilia but believed that the term "basophilic leukemia" should be retained until more complete evidence and confirmation has been presented. He stated that one may consider these numerous basophiles as the progeny of the leukemia myeloblasts potentially basophilic.

Casey, Nettles, and Hidden¹⁵ discussed what has already been stated in this book; namely, whether there is a true basophilic leukemia distinguishable from the neutrophilic type. Doan and Reinhart¹⁶ reported three cases which they believed to be true basophilic leukemia. They seemed to believe that these were the first instances of this sort to be reported.

The case reported by Casey and associates, which records the sixth case in the literature with clinical observations for at least one month before death, emphasized that there is no evidence at present that basophilic leukemia ever

¹¹Groat, W. A., Wyatt, T. C., Zimmer, S. M., and Field, R. E.: *Am. J. M. Sc.* **191**: 457, 1936

¹²Joachim, G.: *Deutsch. Arch. f. klin. Med.* **87**: 437, 1906.

¹³Sabrazès, J.: *Arch. de mal. du coeur* **19**: 38, 1926.

¹⁴Elliott, A. R., and Young, R. H.: *M. Clin. North America* **15**: 69, 1931.

¹⁵Casey, A. E., Nettles, T. E., and Hidden, E. H.: *South. M. J.* **39**: 325-331, 1946.

¹⁶Doan, C. A., and Reinhart, H. L.: *Am. J. Clin. Path.* **11**: 39, 1941.

occurs except as a terminal phase of neutrophilic leukemia. They made a very proper recommendation for future observers; namely, that only those cases should be reported as basophilic leukemia in which (1) blood studies using supravital and peroxidase stains, together with routine blood examinations, have been made; (2) hematologic observations on the patient have been available for more than 3 months prior to death; and (3) post-mortem examination with supravital studies of spreads from bone marrow, blood, spleen, lymph nodes, and alcohol fixation of the tissues for the preservation of the water-soluble basophilic granules have been made.

In a discussion of this paper before the Southern Medical Association, Robert A. Moore came to the same conclusion; namely, that this is a terminal discharge of cells. The disease is basically myelocytic leukemia. He believes also that extreme eosinophilia in myelocytic leukemia does not change the basic diagnosis.

Plasma Cell Leukemia.—

In the literature there have been 36 cases of plasma cell leukemia reported. Foa¹ reported the first case. He called it pseudoleukemia plasmacellularis. He found plasma cells in the blood and diffuse infiltration of the bone marrow, liver, spleen, and in some lymph nodes. Moss and Ackerman² reported a case in September, 1946. This man showed symptoms of swelling of the lower jaw, bleeding gums, enlargement of the spleen and liver. The blood picture showed 13,700 leukocytes, with a differential count of 4 per cent myelocytes, 14 juveniles, 15 stabs, 24 segmented neutrophils, 23 lymphocytes, 6 monocytes, 2 eosinophiles, and 1 per cent basophiles, with 11 per cent plasmacytes, 4 per cent pronormoblasts, and 24 per cent normoblasts. The sternal bone marrow showed the marrow completely replaced by plasma cells. Diagnosis of plasma cell leukemia was made. Plasma cells reached a maximum value of 22 per cent. There was a hyperglobulinemia throughout the disease. Autopsy one and one-half months after admission to the hospital showed splenic enlargement, liver enlargement, and microscopically, plasma cells present in every region of the liver. Spleen sinuses were plugged with plasma cells. Lymph nodes showed invasion of plasma cells, likewise the pancreas. Both marrow showed a complete replacement of normal elements by a diffuse infiltration of plasma cells.

In reference to clinical findings in this disease, it is usually found between the ages of 45 and 60. In contrast to multiple myeloma* in which 70 per cent are males, neither sex shows a predisposition for plasma cell leukemia. The early symptoms of plasma cell leukemia in the majority of cases are those of multiple myeloma. Pain in the arm, back, legs, or substernal region may be present, with a history of lassitude, weakness, and weight loss. There is a tendency to hemorrhage. Bleeding is nearly always from the gums and nose. Bone destructive nature of plasma cell leukemia is exemplified by frequent pathologic fractures. In the bone marrow there is diffuse infiltration with

*Refer to page 927.

¹Foa, P.: Mem. d. r. Accad. sc. Torino 52: 259, 1902. Abst. Folia haemat. 1: 166, 1904.

²Moss, W. T., and Ackerman, L. V.: Blood 1: 5, 396-406, Sept., 1946.

plasma cells. These cells do not differ from those in the blood, except that in the blood the plasma cells tend to be smaller. Liver is invaded by plasma cells, likewise the spleen and lymph nodes.

Differential Diagnosis.—Askanazy³ formulated the following as requirements for a diagnosis of plasma cell leukemia:

1. Plasma cells must be present in the peripheral blood.
2. Leukocytosis.
3. Diffusely involved bone marrow.

A bone marrow biopsy showing diffuse plasma cell infiltration, together with finding the typical cell in the circulating blood, is necessary and usually sufficient. Plasma cells may increase to 30 per cent in measles, in rubella, rubeola, scarlatina, in chronic lymphocytic leukemia, and occasionally in myelocytic leukemia, metastatic carcinoma, and Hodgkin's disease.

On the other hand, bone marrow infiltration by plasma cells unaccompanied by more than occasional plasma cells in the circulating blood may be found in hepatic cirrhosis, agranulocytosis, and miliary tuberculosis.

Laboratory Findings.—In plasma cell leukemia the leukocyte count is usually 10,000 to 40,000. The percentage of plasma cells found in the peripheral blood varies from 87 per cent to 1 per cent. Erythrocyte count is low. Anemia follows the same pattern as in other leukemias. There is a low blood platelet count which is reflected in the high percentage of hemorrhagic tendencies found. Just as in multiple myeloma, hyperglobulinemia is sometimes present in plasma cell leukemia.

Bence-Jones Bodies.—The test for Bence-Jones bodies in the urine was performed in 20 of the 37 cases of plasma cell leukemia. Six of these were positive and the rest negative. A positive test is good evidence of multiple myeloma, for it is infrequent that Bence-Jones bodies are found in diseases other than multiple myeloma.

Although a series of transitions from typical multiple myeloma² to typical plasma cell leukemia has been arranged by Piney and Riach,⁴ and has been discussed by numerous workers,^{5, 6, 7, 8} and the entire series of cases reviewed might be classified more or less in order from those typical of multiple myeloma to those which resemble typical leukemia, it is doubtful if such a list would contribute to knowledge of the disease.

If, as Patek⁷ implied, the more nearly plasma cell leukemia resembles a typical leukemia, the more malignant the disease, the readiness with which plasma cells tend to enter the blood stream should be a measure of malignancy. This seems logical, but it has not been proved. In multiple myeloma the average duration of life after recognition of symptoms is 2 years,⁹ while in plasma cell leukemia it is 8 months. Cases of plasma cell leukemia with an initial plasma cell count in the peripheral blood of over 50 per cent (4 cases) had an

³Askanazy, M., and Dubois-Ferriere, H.: *Helvet. med. Acta* 9: 427, 1942.

⁴Piney, A., and Riach, J. S.: *Folia haemat.* 40: 37, 1931.

⁵Jackson, J., Parker, F. J., and Bethea, J. M.: *Am. J. M. Sc.* 131: 169, 1931.

⁶Osgood, E. E., and Hunter, W. C.: *Folia haemat.* 52: 369, 1934.

⁷Patek, A. J., and Castle, W. B.: *Am. J. M. Sc.* 191: 788, 1936.

⁸Kellhack, H., and Linck, K.: *Deutsches Arch. f. klin. Med.* 188: 88, 1941.

⁹Geschickter, C. F., and Copeland, M. M.: *Arch. Surg.* 16: 807, 1928.

average duration of life of 5 months, while those with an initial plasma cell count under 20 per cent (15 cases) had 8 months' duration. Patek concluded that with such a small number of cases, not much significance can be attached to these figures, but it does suggest that the readiness with which plasma cells enter the peripheral circulation is a measure of the malignancy.

Since multiple myeloma is a leukocytic tumor, the occurrence of leukemia in certain cases brings up again the close relationship between the leukocytic neoplasms and leukemia, the latter probably being a generalized variant of the former condition.

Aleukemia or Leukemia Without Leukocytosis

This class is so-called from its histologic and pathologic-anatomic symptoms. There are anemia, enlarged glands, and splenic tumor. These cases may show a leukemic blood picture with almost a *normal total count*. Aleukemic conditions may be temporary or may change into manifest leukemia. Pinkus believes that the lymphocytic aleukemia is the most common variety. The blood picture rarely approaches normal. There is a relative and sometimes an absolute lymphocytosis with normal or high normal counts. According to Schilling, there is a high-grade regenerative shift. There are 60 per cent lymphocytes, with occasional lymphoblasts. These cases are characterized by lymphoblasts, atypical lymphocytes, Rieder forms with lobulated nuclei, in conjunction with signs of a severe anemia.

Many authoritative sources have reported cases of leukemia without leukocytosis and without enlargement of the spleen. Hirschfeld,⁴ for instance, reported four cases of leukemia without splenomegaly. Ordway and Gorham⁵ reported one case, and Kracke and Garver⁶ also described one case. Weber⁷ reported one case of aleukemic myelosis without splenic enlargement in which the condition clinically simulated aplastic anemia. He believes that many cases, in which the clinical diagnosis was aplastic anemia, were undoubtedly examples of aleukemic myelosis. In other words, we can now speak of the condition called aleukemic myelosis, which is leukemia without leukocytosis and without enlargement of the spleen. Mettier and Purviance⁸ reported five cases of leukemia in patients who had no leukocytosis and no splenic enlargements. The clinical findings were those of severe anemia, thrombopenia, and leukopenia with the presence of immature forms of leukocytes in the stained films of blood.

The first case showed 7,600 leukocytes per cubic millimeter with a differential count of 39% segments, 2% nonsegments, 45% lymphocytes, 3% monocytes, 4% myelocytes, 5% myeloblasts, and unclassified immature forms, 2%. One nucleated red blood cell was seen per hundred white blood cells. There was a diminution in the number of blood platelets.

The patient presented the clinical symptoms of a severe blood dyscrasia and died following hemorrhage from the bladder and gastrointestinal tract. The clinical diagnosis was aleukemic myelosis with slight leukemic metaplasia of the cervical lymph nodes. Section

⁴Hirschfeld, H.: *Ztschr. f. klin. Med.* 80: 126, 1914.

⁵Ordway, T., and Gorham, W.: *The Diagnosis and Treatment of Diseases of the Blood*, in Christian, H. A.: *Oxford Monographs on Diagnosis and Treatment*, London, 1930, Oxford University Press, Vol. 9.

⁶Kracke, R. R., and Garver, H. E.: *Internat. Clin* 4: 37, 1935.

⁷Weber, F. P.: *Quart. J. Med.* 1: 409, 1932.

⁸Mettier, S. R., and Purviance, R.: *Arch. Int. Med.* 60: 458, 1937.

of this cervical lymph node showed that the architecture of the lymph node had been largely replaced by tumor cells of various sizes, mostly of the myeloid type. There were myelocytic cells of the neutrophilic and eosinophilic types present.

A second case was a 38-year-old white man who showed 2,300 leukocytes or a very definite leukopenia, with a differential count of 7% polymorphonuclears, 22% nonsegmented neutrophils, 42% lymphocytes, 26% monocytes, 1% myelocytes, and 2% unclassified cells. Two nucleated red blood cells were seen per hundred leukocytes in the stained film. Blood platelets were found greatly reduced. This patient showed anemia and leukopenia with no apparent hematopoietic reaction to liver extract given either parenterally or by mouth. After a number of weeks the patient returned to the hospital, from which he had been discharged, with the same picture with 20% myelocytes, and 16% myeloblasts, and 40,000 platelets per cubic millimeter. Clinical diagnosis was aleukemic myelosis with anemia and thrombopenia.

There were several other cases reported of similar type.

It is interesting to note that the biopsy of the bone marrow from the sternum afforded a valuable aid in establishing the diagnosis. Specimens of the marrow showed a predominance of undifferentiated types of cells, many of which contain mitotic figures, packed solidly together, to the exclusion of fat cells. This picture of the bone marrow indicated a hyperplasia which was undoubtedly leukemic.

Diseases Simulating Leukemia

Lymphosarcoma.—This term has been used loosely to cover all neoplastic states of lymph nodes that are not clearly Hodgkin's disease or leukemia. Strictly speaking, lymphosarcoma is a disease in which there is a neoplastic transformation of the lymphocytic strain of cells. Hematologically lymphosarcoma may show no detectable changes in the peripheral blood except perhaps a secondary anemia or an occasional low-grade lymphocytosis, or both. The neoplastic cells may break over into the blood stream in greater or lesser number, producing a type of leukemia first described by Sternberg⁹ as leukosarcoma. Wiseman records the results of a series of blood examinations made in a case of lymphosarcoma in which the tumor type of cell (in contrast to normal lymphocytes) was found in the blood stream only intermittently. This case, histologically, answered the description of the tissue described by Brill, Baehr, and Rosenthal¹⁰ under the term "follicular lymphoblastoma" and is the most chronic type of leukosarcoma encountered.

Chloroleukemias.—This is a disease described by Naegeli. It displays an acute leukemic blood picture. The symptoms are cachexia, severe anemia, and subperiosteal circumscribed proliferations, manifested principally on the skull.

Leukosarcomatosis.—This was classified by Sternberg as a separate group. These cases show the picture of acute leukemia of the myelocytic and lymphocytic varieties with highly infiltrated tumor growths.

Pseudoleukemia.—This term is a misnomer and should be replaced by the term "apparent leukemia." These cases fall into the following: (1) malignant granuloma or lymphogranulomatosis, also called Hodgkin's disease; (2) generalized tuberculous granuloma of the lymph glands; (3) generalized syph-

⁹Sternberg, C.: Ueber Leukosarkomatose, *Wien. klin. Wchnschr.* 21: 475, 1908.

¹⁰Brill, N. E., Baehr, George, and Rosenthal, Nathan: *J. A. M. A.* 84: 668, 1925.

ilitic granuloma; (4) myelogenous metaplasia through osteofibrosis, the so-called "marble" disease, tumors of the bone marrow with myelogenous enlargement of liver, spleen, and glands.

Lymphogranulomatosis Maligna—Hodgkin's Disease

This disease was described by Hodgkin in 1832 in a publication entitled, "On Some Morbid Appearances of the Absorbent Glands and Spleen."¹ Many observations have been made since that time. Considerable experimental work has been done, but the etiological agent has not yet been discovered. This disease was once called "pseudoleukemia." Bonfils in 1856 spoke of it as a cachexia without leukemic changes. Virchow in 1865 classified it with the lymphosarcomata. Cohnheim, unfortunately, introduced the term "pseudoleukemia," but this has been proved to be manifestly an incorrect classification. Billroth in 1869 called it "malignant lymphoma"; Langhans in 1872, "malignant lymphosarcoma"; Schulz in 1874, "reticulated desmoid carcinoma"; Orth in 1887, "malignant aleukemic lymphadenoma"; and Langebeck termed it "gland sarcoma." Many regard it as an infectious granuloma due to a specific microorganism, although this microorganism has not yet been identified.

So far as predisposing causes are concerned, it seems to occur in males three times as frequently as in females. Age incidence is a well-defined factor. The preponderance of cases occurs in young adults and in middle-aged individuals, one-third appearing before the twentieth year and one-half between the twentieth and fortieth years.

Geographically, it occurs in all parts of the world, sparing no race. There are no data on hand to show any etiological basis from the standpoint of family predisposition. It is striking that the greater number of cases appear in individuals of robust constitution, previously in apparently good health. The disease is likely to occur among the rich as well as among the poor. There is no information at hand to show that any previous disease plays a role in the excitation of this disease.

Some believe in the infectious nature of this disease because of the histopathologic evidences found in the affected tissues. The positive character of this evidence is the resemblance exhibited by the changes produced in the glands to those caused by other infectious diseases, particularly syphilis and tuberculosis. There is great dissimilarity between this disease and the clinically similar leukemic and lymphosarcomatous lymph node enlargements.

Many organisms have been described as the etiological agents of this disease but none have stood the test of time. Some have claimed that the tubercle bacillus causes Hodgkin's disease; others that it is due to the pseudodiphtheria bacillus; and even the "bacterium hodgkini" has been ardently brought forward as the causative bacteriologic agent of this disturbance.

Wise and Poston* allude to the coexistence of brucellosis and Hodgkin's disease, which is in line with those who believe in the infectious origin of this disease. *Brucella* were found in fourteen cases of Hodgkin's disease by isolation of *Br. melitensis* by blood or lymph gland culture.

*Wise and Poston: J. A. M. A. 115: 1976, 1940.

¹Hodgkin, T.: Med.-Chir. Tr. 17: 68, 1832.

Summarizing, the condition is truly a granuloma of unknown origin. It may be bacterial or virus in origin. According to the view of Brill and Symmers, it holds an intermediate position between an infectious granuloma and a true tumor. Ewing's work points to the transition of Hodgkin's granuloma into a sarcoma, although Krumbhaar in a large series of cases with autopsy findings does not believe that Hodgkin's disease can develop into a Hodgkin's sarcoma.

Symptoms.—Trousseau has divided the clinical course of Hodgkin's disease into two phases: (1) that of the local enlargement of the lymph nodes, known as the "latent period"; and (2) that of the "generalization" of the process with the involvement of the lymph glands generally, of the spleen in most cases, and of other organs, and of progressive cachexia. The latent period is characterized by the patient's noticing something wrong with his appearance, such as a growth on one side of the neck, which is not painful, is not tender, and in no way seems to affect the general health. Sometimes in the case of nonobservant individuals, the patient presents himself only after a rather enormous enlargement at the side of the neck has developed. The duration of the so-called "latent" period is variable, depending upon the virulence of the infection and upon the resistance of the individual. It may vary from several weeks to four years or more.

The second period of Trousseau witnesses the "generalization" of the process, the involvement of other lymphatic structures, and of other organs, febrile phenomena, and the appearance of anemia and cachexia. The lymphatic gland enlargement, which began in the neck, extends to the opposite side, is followed by enlargement of the axillary, inguinal, and other superficial groups; this in turn is followed by involvement of the deeper glands. Spleen and liver enlargement with signs of pressure phenomena exerted upon the lungs, pleurae, hilus of the liver, nerve trunks, and veins occur. Fever is present followed by changes in the blood, but these blood changes are not characteristic.

Serious involvement of the central nervous system occurs in this disease. The changes in the nervous system are damage to the spinal cord, due to direct pressure caused by extradural deposits of the abnormal granulomatous tissue by disease on one or more vertebral bodies with consequent angulation, and also a degeneration resulting from interference with the blood supply of the cord. Actual inflammatory and degenerative changes in the cord substance have been observed by Forrest,¹ Shapiro,² Weber,³ and Weil.⁴ A later observation on the invasion of the spinal canal by lymphogranulomatosis maligna is that by Cooper.⁵ He reported a case of lymphogranulomatosis maligna with invasion of the spinal canal and concluded that nerve roots afford a route by which lymphogranulomatous tissue may reach the subdural space, probably through the lymphatic spaces of the roots rather than

¹Forrest, Devereux: *Lancet* 2: 809, 1927.

²Shapiro, P. F.: *Arch. Neurol. & Psychiat.* 24: 509, 1930.

³Weber, F. P.: *Internat. Clin.* 1: 126, 1926.

⁴Weil, Arthur: *Arch. Neurol. & Psychiat.* 20: 1009, 1931.

⁵Cooper, M. J.: *J. A. M. A.* 102: 917, 1934.

by the substance of the nerve fibers. The spinal dura mater is relatively more resistant to penetration than are the septums of the nerve roots. Paraplegic symptoms may result not only from direct cord compression but from obstruction to the circulation of the cord as a result of compression or invasion of the vessels accompanying the nerve roots.

In addition to the lymphatic gland enlargement, the spleen is usually involved with moderate enlargement, although enormous tumors are occasionally observed. Sometimes, enlargement of the tonsils is noted. Most of the circulatory symptoms are due to compression. The liver often participates in the process. Clinical symptoms are the enlargement of the organs. Jaundice is sometimes seen, due to compression of the common duct by the lymphomatous masses. The cause of diarrhea, which is commonly found in this disease, is not known, but it is probably a toxic manifestation. Nodules are sometimes seen in the osseous system. There are two types of symptoms in the cutaneous manifestations, the one due to the specific process, consisting of the deep-seated, destructive changes made up of the characteristic granulation tissue of the disease, and the other in all probability also specific and consisting of the superficial, ephemeral lesions which are probably of toxic origin. Pruritus occurs in about 10 per cent of the cases; in association with the diarrhea and the lymphatic gland enlargement, it is of considerable diagnostic importance. Pigmentation is another cutaneous symptom sometimes occurring as the result of the itching lesions, but frequently not associated with pruritus and of unknown cause.

The Blood Picture.—The hematologic picture of this disease has been a subject that has been much discussed. *Certain it is at this time that there is no characteristic picture in the blood by which one can identify the disease as Hodgkin's disease.* There are changes, it is true, but they are not characteristic. The red cells are reduced in number and there is usually a diminution in the percentage of hemoglobin. The red cell count may vary from 2 to 4 million, but sometimes drops to the million mark. The red cells are pale and there are often many microcytes. Occasionally, nucleated red cells are seen, together with poikilocytosis, polychromasia, and basophilic punctation. The number of white cells varies. Occasionally Hodgkin's disease displays an unusual increase in the number of white cells, but this increase is not a characteristic phenomenon. There may be a leukocytosis as well as a leukopenia. McAlpin¹ found the cell count ranged in eighteen cases between 2,000 and 13,000.

Ziegler² in seventy cases found the following: in 22, counts of from 5,500 to 10,000; in 20, from 10,000 to 20,000; in 8, from 20,000 to 30,000; in 2, more than 30,000; and in 18, a leukopenia of from 2,000 to 5,500. In a general way, there is usually in the early stages of the disease a slightly increased total count, or perhaps a normal count. The differential count has been much discussed but as yet no one has had the temerity even to suggest that there is any possibility of making a diagnosis of Hodgkin's disease from the differential count. Early in the disease there may be a lymphocytosis, but this is not positive. A leukopenia with a relative lymphocytosis has been described in

¹McAlpin: Arch. Int. Med. 33: 954, 1933.

²Ziegler: Hodgkin's Disease, Jena, 1911.

the beginning of the disease. Much emphasis has been laid upon the *eosinophilia* in Hodgkin's disease, but this has been denied by others. Our own experience has shown only a slight eosinophilia, but that the eosinophilia in the blood runs parallel with a tissue eosinophilia admits no denial. This may be due to the fact that the chemotactic influences exerted by the specific granulation tissue upon the bone marrow bring in response not only the number demanded by the tissues but, as is usual, an excess, which enters the circulating blood and accounts for the eosinophilia. *The eosinophilia, if present, is of diagnostic importance, but if absent, is not sufficient to exclude this disease.*

A very interesting observation has been made by the author in three cases of Hodgkin's disease. This observation is offered here not as a diagnostic measure but as a pure matter of record. In three different independent cases observed in three years, we have noted a rather peculiar condition of the red cells in Hodgkin's disease. Blood diluted with Hayem's solution for the red cell count has shown distinct clumping of the red cells, making it rather difficult in these cases to perform an accurate red count. The blood in these cases when diluted with 2.5 per cent sodium citrate solution did not display any clumping. The fact that this was observed in three independent cases of clinical and pathologic Hodgkin's disease certainly deserves mention. So far as we know, it has not hitherto been mentioned in the literature.

The actual diagnosis of Hodgkin's disease can be made only by excision of the lymphatic gland and histopathologic study. The characteristic picture of the lymphatic gland in this disease is as follows: first, a partial replacement of the normal lymphatic tissue by a new growth of connective tissue; second, the finding of rather typical cells (see Fig. 208) which have been well described by Reed in her studies on "The Pathological Changes in Hodgkin's Disease."*

In addition to the new growth of connective tissue, there are endothelial and reticulum cells, the formation of characteristic giant cells and often a large number of eosinophiles.

The Diagnosis of Hodgkin's Disease by Aspiration Biopsy

Biopsy by means of aspiration through a needle has been repeatedly described.¹⁻⁵ Loseke and Craver⁶ described their five years' experience in establishing the diagnosis of Hodgkin's disease and related diseases by means of aspiration biopsy. Their method is to aspirate tissue into the bore of a hollow needle.

Method.—

Accurately localize the site within the tumor into which the point of the aspirating needle is to be inserted.

Prepare the overlying skin by shaving, if necessary, and with iodine and alcohol.

With 1% Novocain anesthetize a wheal at the site in the overlying skin selected as the appropriate place for introduction of the needle.

*Reed, D.: Johns Hopkins Hosp. Rep. 10: 133, 1902.

¹Craver, L. F.: Surgery 8: 947-960, 1940.

²Idem, and Binkley, J.: J. Thoracic Surg. 8: 436-463, 1939.

³Martin, H. E., and Ellis, E. B.: Ann. Surg. 92: 169-181, 1930.

⁴Idem: Surg., Gynec. & Obst. 59: 578, 1934.

⁵Pavlovsky, Alfredo: Thesis, Buenos Aires: A. Lopez, 1934.

⁶Loseke, L., and Craver, L. F.: Blood 1: 1, 76, Jan., 1946.

Make a tiny incision clear through the skin with a No. 11 Bard-Parker bistoury, so that elastic fibers of the skin will exert no drag on the needle.

Carefully introduce an 18-gauge needle (accurately fitted with a stylet, and having a rather short bevel) through the incision in the anesthetized skin until the point of the needle reaches the desired site within the tumor.

Remove the stylet and attach a 20 c.c. Record syringe to the needle. Draw the piston back so as to create a strong negative pressure within the needle-syringe system. While this negative pressure is maintained, slightly advance and withdraw the needle a few times, sometimes with rotation. If negative pressure is partially lost by leakage, detach the syringe and expel the air from it; then reattach it and again produce negative pressure.

When soft tumors are being aspirated, from 0.5 to 3.0 c.c. of bloody material may be sucked into the syringe. Firm or hard tumors may yield only a tiny bit of tissue, found within the bore of the needle. Occasionally nothing significant will be obtained, and the procedure may have to be repeated, if possible.

Allow the pressure within the syringe to become nearly normal, so that when the needle is withdrawn there will be no spattering of blood and tissue inside the syringe; then withdraw the needle.

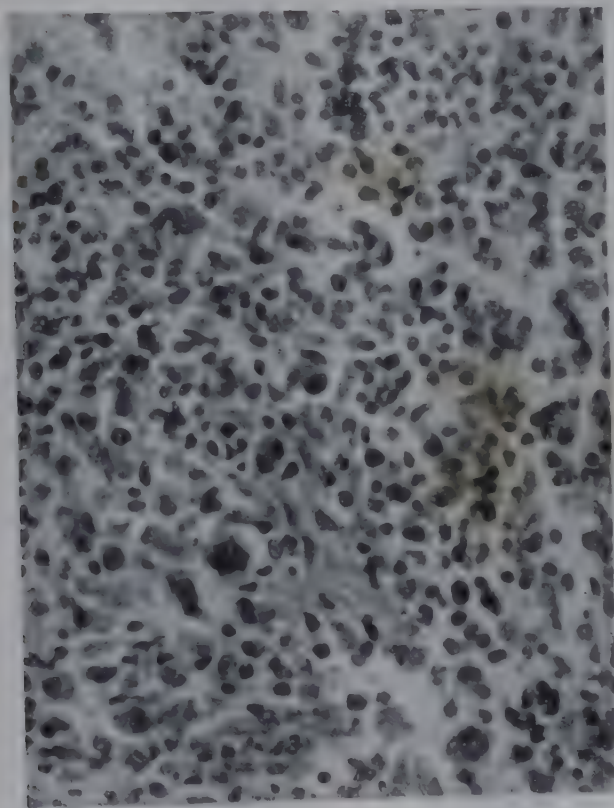


Fig. 205.—Case 1. Section of clot obtained from infiltrated portion of left upper lobe of lung by aspiration biopsy. Diagnosis of Hodgkin's disease was made by means of this section confirmed clinically by subsequent course. (Loseke, Lucile, and Craver, Lloyd F.: *Blood*, J. Hematol. 1: 1, 1946. Courtesy Grune & Stratton, Inc., New York.)

Allow any material collected within the syringe to clot, if it will. Remove the clot, place on a small square of blotting paper, and then drop into a small "biopsy" bottle containing 10% Formalin. Prepare paraffin blocks, as for any tissue biopsy, and section.

Push out material in the needle by means of the stilet and blow onto slides. Other fragments may be found inside the syringe walls or on the face of the piston. Crush the material firmly and spread between slides; quickly stain with hematoxylin and eosin. This procedure is known as the "immediate spread."

The procedure used when a tumor within the chest is aspirated does not differ essentially from that described above.

Loseke and Craver⁶ found that in a study of 242 cases of histologically proved Hodgkin's disease, the diagnosis was established by open biopsy in 228, while in 14 cases aspiration biopsy yielded a reliable diagnosis.

⁶Loseke, L., and Craver, L. F.: *Blood* 1: 1, 76, Jan., 1946.

It is interesting to note that in cases of Hodgkin's disease without enlarged peripheral lymph nodes, yet presenting nodes or masses accessible to needle puncture, the method of aspiration biopsy has often proved successful in establishing the diagnosis. Success depends in large measure on examination, by an experienced pathologist, of a sectioned clot from the aspirated tissue.

Diagnosis of Case 1⁶ was made from Fig. 205. This was a case of infiltration in the anterior part of the left upper lobe of the lung. It might have been tuberculosis, bronchogenic carcinoma, or Hodgkin's disease. There were no enlarged peripheral nodes. Aspiration biopsy of the lung gave an immediate spread which yielded insufficient tissue, but the clot showed Hodgkin's disease.

The case illustrated in Fig. 206 was the fourth clinical case reported by Loseke and Craver.⁶

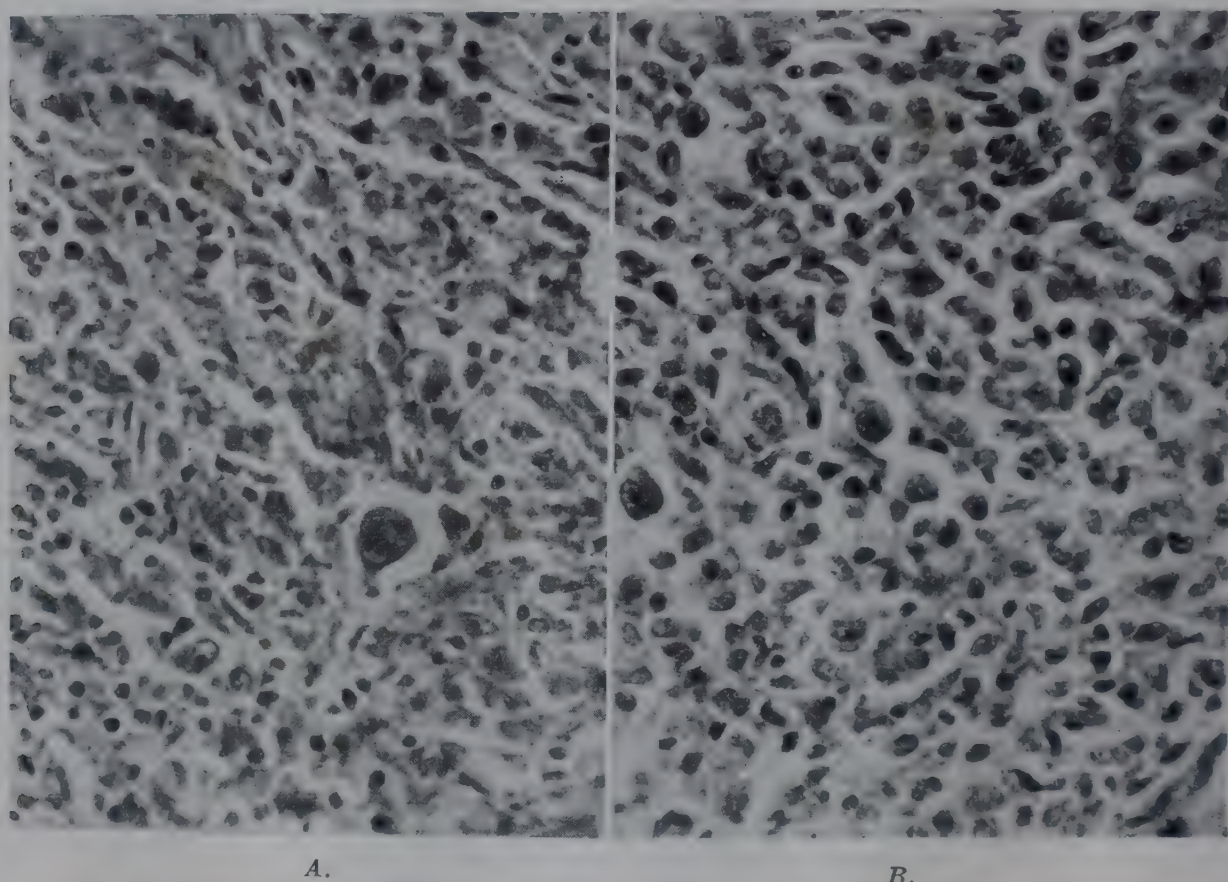


Fig. 206.—Case 4. A. Aspiration biopsy from spleen before any nodes were available, showing Hodgkin's disease.

B. Section of surgical biopsy from axillary lymph node two months later, confirming diagnosis obtained by spleen biopsy. (Loseke, Lucile, and Craver, Lloyd F.: *Blood*, J. Hematol. 1: 1, 1946. (Courtesy Grune & Stratton, Inc., New York.)

The onset of illness had occurred two years before, with general severe itching. There had been a weight loss of from 35 to 40 pounds over two years, and night sweats and intermittent fever for one year. Dyspnea and wheezing were present. Widespread excoriation of the skin from scratching, emaciation, and an appearance of chronic illness were found. Spleen was enlarged to the level of the iliac crest. Liver was enlarged by two fingerbreadths below the right costal border. There was roentgenographic evidence of some widening of the superior mediastinum, and of hilar infiltration. Moderate anemia was present. No peripheral nodes were suitable for biopsy. Aspiration biopsy of the spleen was made and the spread gave evidence consistent with the criteria for Hodgkin's disease. The clot showed Hodgkin's disease. Later, an enlarged

⁶Loseke, L., and Craver, L. F.: *Blood* 1: 1, 76, Jan., 1946.

node was found in the left axilla. Biopsy of this node confirmed the diagnosis of Hodgkin's disease. Fig. 206 is a section of surgical biopsy from axillary lymph node confirming diagnosis obtained by spleen biopsy.

The blood picture of a case of Hodgkin's disease observed in 1928 at the Christian Hospital, in St. Louis, was as follows:

Woman, aged 29, showed clinical enlargement of lymphatic glands in the neck and groin.

Red cells, 4,370,000; Leukocytes, 5,600; Hemoglobin, 77%; Color Index, 0.8.

Hemogram as follows:

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
1	1	0	0	4		69	22	3

A second hemogram three days later showed:

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
3	1	0	0	5		76	13	2

The thick drop showed 2+ polychromasia.

The following changes in the erythrocytes were noted: 1 megaloblast and 1 reticulocyte to the field. The Wassermann, Kolmer, Kahn, and Hecht-Gradwohl tests were negative.

Microscopic examination of tissue from gland of the neck showed a lymphatic gland structure with many large cells, evidently Reed cells, many eosinophiles with areas of focal necrosis in the gland. There was marked fibrous connective tissue reaction. Note on this record reads: "We are inclined to believe that this is a case of Hodgkin's disease rather than a lymphosarcoma."

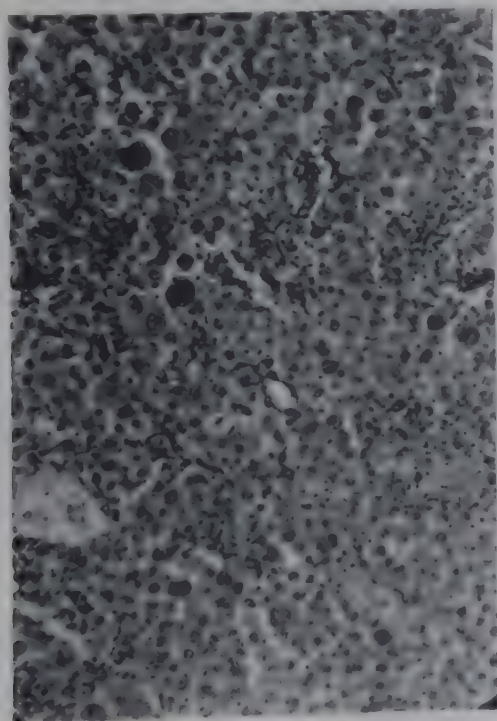


Fig. 207.

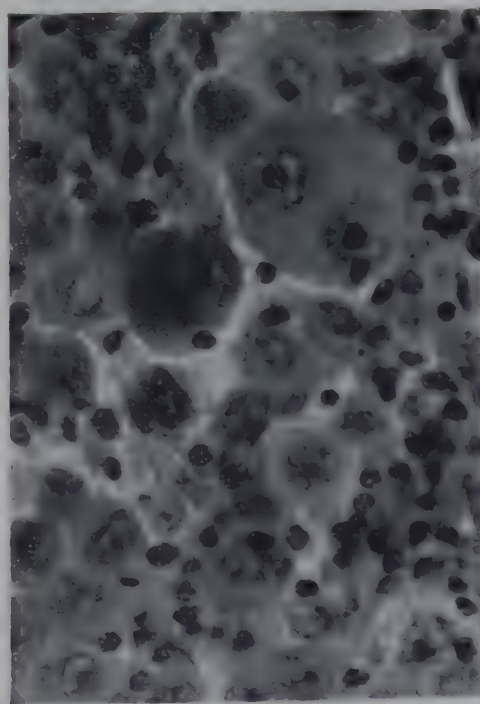


Fig. 208.

Fig. 207.—Section of lymph node, Hodgkin's disease, showing Dorothy Reed cells. Low-power magnification.

Fig. 208.—High magnification of a portion of Fig. 207. Hodgkin's disease, showing Reed cells.

A case observed in 1931—man, aged 62 years.

Marked lymphatic gland enlargement in the neck and a few in the axillary region.

Blood Picture: Erythrocytes 2,130,000. Mention is made in the report that the blood cells clumped with Hayem solution but went into proper suspension with citrate solution.

Leukocytes, 5,475; Hemoglobin, 52%; Color Index, 1.2; Reticulocytes, 6.8%; Blood Platelets, 220,345.

Hemogram as follows:

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
0	2	0	2	18		29	46	3

Thick drop showed 2+ polychromasia.

Wassermann, Kolmer, Kahn, and Hecht-Gradwohl negative.

Microscopic examination of the lymphatic gland from the neck showed typical epithelioid giant cells with a few eosinophiles and a marked reaction of fibrous connective tissue. Diagnosis on this report was "typical structure of gland from Hodgkin's disease."

A case observed in the St. Louis County Hospital is recorded as follows:

Male, aged 10 years. Patient had enlargement of gland on left side of neck for six or seven years, increasing in size gradually until it is now markedly enlarged. This gland seems not to be attached to other parts of the neck, and it can be moved about under the skin. It seems to be cystic in one or two places.

There is no redness, pain, or tenderness. There are no other glands involved.

Patient had no cough, but takes cold easily. Head negative, tonsils enlarged. Heart, systemic murmur. Abdomen, spleen not palpable; no masses; no tenderness. Extremities, negative.

Reflexes, equal and active.

Patient has good appetite, but sometimes is unable to retain food.

About one and a half years ago patient had an ultraviolet ray treatment, but with no apparent improvement.

Since admittance to hospital temperature ranges from 100° to 101.6° F.; pulse ranges 118 to 132; respiration 20 to 26.

X-ray showed chronic bronchitis and cardiac hypertrophy.

Blood Picture: Red cells, 2,630,000 (clumping of red cells noted); Leukocytes, 15,600; Hemoglobin, 40%; Color Index, 0.7.

Hemogram as follows:

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
0	0	0	0	6		82	12	0

Blood Picture five days later showed: Red cells, 3,440,000; Leukocytes, 27,990; Hemoglobin, 42%; Color Index, 0.6.

Hemogram as follows:

Bas.	Eos.	Myel.	Juv.	Stabs		Segs.	Lymphs.	Monos.
0	0	0	0	8		86	5	1

NOTE.—In making the red cell count on March 14, the red cells clumped with Hayem solution but went into proper suspension with 2.5 per cent sodium citrate solution. This was noted again on March 19. However, one blood transfusion was given this patient and on the occasion of the next count of the red cells on March 24, the clumping in Hayem solution was not observed.

Microscopic sections of a lymphatic gland showed some degree of proliferation of the lymphoid cells with considerable invasion of connective tissue which has impinged upon the normal lymphoid tissue area. This obliteration is marked in certain parts of the gland. Scattered among the lymphoid cells are many large cells many times the size of the lymphocytes. Cytoplasm is clear, with irregular stainable shreds. They contain one or two nuclei which are rounded or indented. Some of these nuclei contain nucleoli. Occasionally epithelioid cells are found, the so-called Dorothy Reed cells.

Brill-Symmers Disease (Giant Follicular Lymphadenopathy)

This is a disease which involves the lymph nodes and spleen. It has been designated generalized giant lymph follicle hyperplasia of lymph nodes and spleen, generalized follicular lymphadenopathy with splenomegaly, benign lymphosarcoma, pseudoleukemia, Brill-Symmers disease, Baehr-Brill's disease.

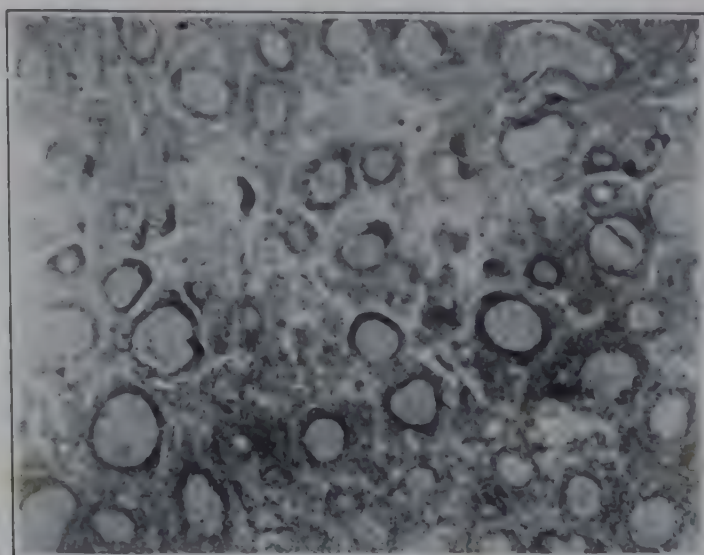


Fig. 209.—A benign type of multiple giant follicular lymphoblastoma. (Courtesy Dr. Walter Siebert, St. Louis, Mo.)

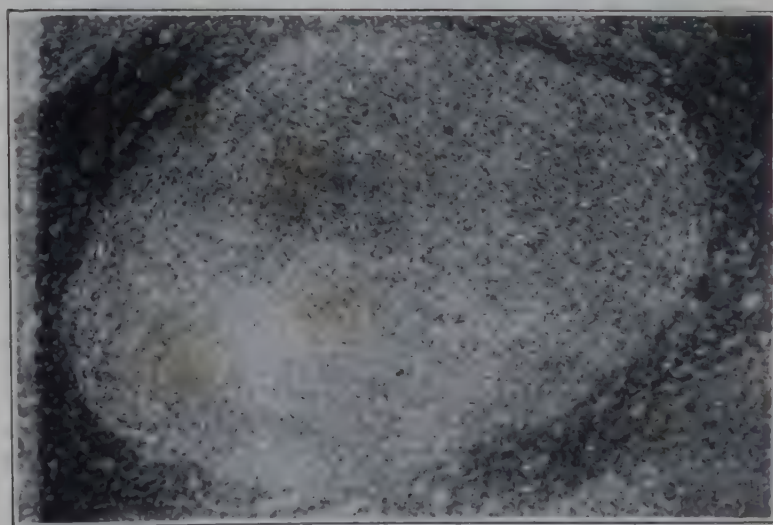


Fig. 210.—High-power enlargement of lymphoblastoma. (Courtesy Dr. Walter Siebert, St. Louis, Mo.)

Clinically it resembles Hodgkin's disease. Microscopically, sections show a replacement of the normal architecture of lymph glands by dimensionally and numerically increased, abnormally shaped lymph follicles due to a peculiar hyperplasia of their germinal centers. Siebert¹ reported five cases. He divided these cases into two groups: (1) in which the hyperplastic follicles in the lymph nodes and spleen maintain their structural identity for a long time, or disappear temporarily, or rupture and heal; (2) in which the case may transform into (a) a polymorphous lymphosarcoma, or (b) become associated with histologic changes of Hodgkin's disease, or (c) with the changes of lymphocytic leukemia, or (d) with those of a disease with necrotic lesions, or (e) a combination of giant follicular lymphosarcoma and Hodgkin's disease which may be seen in the same lymph nodes.

¹Siebert, W. J.: Bull. St. Louis Med. Soc. 36: 415, 1942.

Clinical Symptoms.—Lymph nodes become enlarged, remaining discrete, may become as large as 10 cm. There is usually weakness, anorexia, loss in weight, and low-grade fever.

Blood Picture.—Ten per cent of the cases show a mild leukopenia, with a lymphocytosis of from 50 to 60 per cent, and a mild eosinophilia, 4 to 6 per cent. There may be a slight anemia.

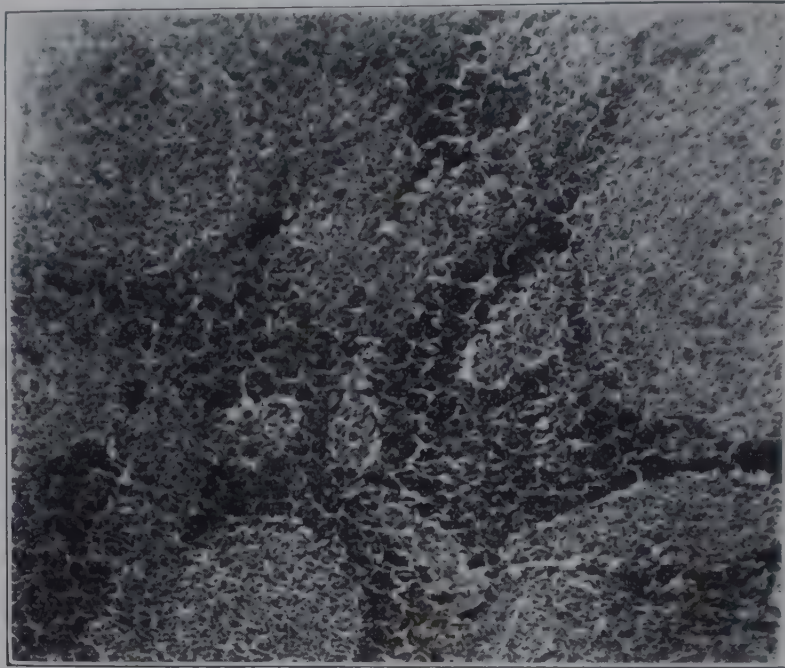


Fig. 211.—Low-power view of early transformation of the giant follicular blastoma into polymorphous cell lymphosarcoma. (Courtesy Dr. Walter Siebert, St. Louis, Mo.)

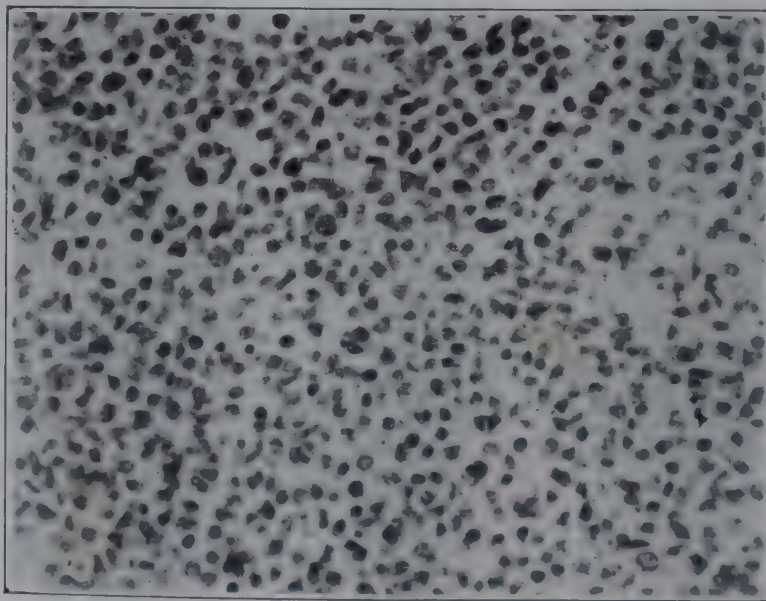


Fig. 212.—High-power view of polymorphous cell lymphosarcoma arising from a giant follicular blastoma. (Courtesy Dr. Walter Siebert, St. Louis, Mo.)

The disease is important because it is likely to be confused with other conditions. Biopsy of the glands should be sufficient to clarify the situation.

Cervical Lymphadenopathy

This group of diseases is due to various causes. One may divide all lymphadenopathies, cervical or otherwise, into three groups, according to Wiseman¹: (1) those in which the predominant characteristics are significant

¹Wiseman, B. K.: Ohio M. J. 29: 364, 1933.

of malignancy; (2) those in which the reaction is like an infectious process; and (3) those which seemingly bear no relationship to either infection or neoplasm.

The first group is composed of three clinical subdivisions: (1) lymphosarcoma, including those tissues having the pathology typical of lymphocytoma, lymphoblastoma, reticulum cell sarcoma, or plasmacytoma; (2) the leukemias, comprising the chloromas, mixed-cell leukemias, leukosarcomas, pseudoleukemias, and the aleukemias as well as the pure myelocytic, lymphocytic, or monocytic leukemias; and (3) true tumor metastases in which group is included not only the cancer metastases but also the primary lymph node endotheliomas.

The second major groups, the infectious, nonmalignant lymphadenopathies, comprise four clinical types: (1) lymphatic tuberculosis, (2) syphilis, (3) simple lymphadenitis (infections of focal origin), and (4) infectious mononucleosis.

The third major group, comprising diseases which are neither infectious nor malignant, is represented by only one class, lymphadenosis, or benign hypertrophy.

Differential Diagnosis.—At this point the knowledge of the regional anatomy of the lymphatic tissues is indispensable in differential diagnosis; for instance, enlargement of the preauricular node may be due to a focus of chronic conjunctivitis. In order to make a correct diagnosis, one must consider the following points: temperature, characteristics of the nodes, splenomegaly, blood picture, and biopsy.

Fever is found only among the diseases of the infectious group.

With respect to the characteristics of the nodes, the presence or absence of pain, the distribution of the swellings, whether local or generalized, and third, the physical characteristics imparted to the palpating fingers by the diseased nodes, are points to be determined. Painful glands suggest infection, which may be inflammatory.

Distribution of the gland swellings is important. Thus, generalized adenopathy without fever is characteristic of the chronic leukemias, the afebrile stage of Hodgkin's disease, and the benign hypertrophies; if fever is present, a generalized swelling means syphilis, Hodgkin's disease, infectious mononucleosis, generalized lymphatic tuberculosis, serum sickness, or an acute phase of leukemia. Local swelling without fever suggests lymphosarcoma, tumor metastasis, lymphadenosis, tertiary syphilis, or Hodgkin's disease. Local swelling combined with pain and inflammation is nearly always indicative of local infection, either lymphatic tuberculosis or pyogenic lymphadenitis, but never of Hodgkin's disease.

Palpation gives important data. Some cases of metastatic carcinoma and some very chronic types of Hodgkin's disease show very hard and unyielding glands but many do not. Many malignant adenopathies and cases of Hodgkin's disease exhibit discrete, not fused, glands. In leukemia, the glands are always discrete and elastic to the touch. A chronic fluctuant or soft node, if painful, adherent to the skin, or showing evidence of previous sinus infection, usually means localized lymphocytic tuberculosis.

So far as enlargement of the spleen is concerned, a hard spleen extending nearly to the umbilicus with concomitant generalized adenopathy nearly always means leukemia—and the larger the spleen the more likely is the leukemia to be myelocytic in type. In Hodgkin's disease the spleen does not usually extend much below the costal border and is not palpable at all in one-half of the cases. A slightly enlarged spleen is seen in infectious mononucleosis.

The Blood Picture.—In malignant cervical adenopathies, the blood picture is normal except in those patients in an advanced stage or with leukemia. Leukocytosis with neutrophilia is strong evidence that the adenopathy in question is of infectious origin. With lymphocytosis, monocytosis, and mild anemia of the hemoglobin type, we suspect lymphatic tuberculosis; with lymphopenia, eosinophilia, and monocytosis, and usually leukopenia, with marked anemia of the red blood cell type, the blood reflects Hodgkin's disease. Normal total reds and whites, with eosinophilia and lymphocytosis, suggest syphilis; marked lymphocytosis, Rieder cells, and normal red cells suggest infectious mononucleosis. With simple pyogenic lymphadenitis, there is only a neutrophilia. Noninfectious, nonmalignant lymph gland diseases are characterized by normal total counts but relative increases in the lymphocytes. Do not confuse this group with aleukemic phases of chronic lymphocytic leukemia.

Biopsy.—Select the gland that is most likely to contain the most pathology; i.e., usually the largest gland. Remove the entire tissue, but do not cut across nor rupture the gland.

“Significant” Inguinal Lymphadenopathy—“Triangle” Adenopathy of Agress

Lymphadenopathy in the malignant lymphomata and leukemic diseases is so well known that it hardly appears necessary to elaborate on it. However, a number of years ago, we were impressed with the distribution of lymph node enlargement in the *inguinal* region in these conditions. When lymph nodes were present in the groin in the malignant lymphomata, they often extended down to the apex of Scarpa's triangle. By recurrent local usage, such nodes have taken on the designation of “triangle” adenopathy in contradistinction to “inguinal” adenopathy located in closer proximity to the inguinal ligament (Fig. 213). We have been unable to find any reference in the literature suggesting that there is any clinical significance in distribution of inguinal nodes, albeit there are general references to inguinal adenopathy in malignant disease.^{1, 2, 3} Wintrobe's¹ Fig. 197 shows this triangle adenopathy in an extreme case of “reticulum cell” sarcoma, but he makes no special point about the distribution.

In an attempt to elaborate this physical sign, we examined 494 normal and abnormal subjects for inguinal adenopathy. The examinations of all individuals in this study were carried out with the patient in the supine position. The inguinal regions were inspected with the extremities outstretched

¹Wintrobe, M. M.: *Clinical Hematology*, Philadelphia, 1952, Lea & Febiger, pp. 818, 889.

²Sugarbaker, E. D., and Craver, L. F.: *J. A. M. A.* 115: 17, 112, 1940.

³Taylor, G. W., and Nathanson, I. T.: *Lymph Node Metastases*, New York, 1942, Oxford University Press.

and on occasion adenopathy was readily visible. Following palpation in this position, the thigh was flexed and slowly abducted and adducted. This latter maneuver accentuated the boundaries of Scarpa's triangle and permitted ready palpation of the apical and deeper portions of this area. The remainder of the lower extremity, the buttocks, and the lower abdominal wall were then examined for infection, and the subject was questioned concerning infection in these regions.

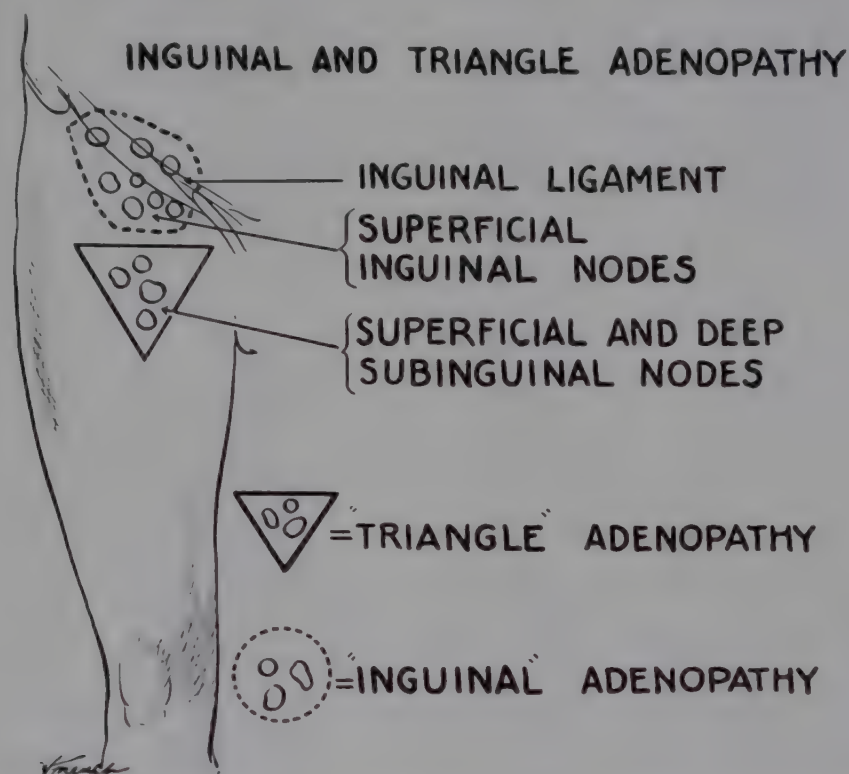


Fig. 213.—Inguinal and triangle adenopathy. (Contributed by Dr. Harry Agress.)

The control group of this report consisted of 256 subjects ranging in age from 10 to 82 years. All of these patients were seen either for routine examinations or for disease processes not generally associated with lymphadenopathy. For the purpose of this study, these patients can be considered "normal." Fifty-four (21.0 per cent) of these normal subjects displayed "inguinal" adenopathy (Fig. 214 and Table 68). This category included every individual who showed even the smallest palpable node, the degree of adenopathy being almost universally of the variety familiarly noted in records as "no significant adenopathy" or "small shotty nodes." In all instances these were felt only in the immediate vicinity of the inguinal ligament and in *no case* were nodes felt *extending down to the apex of Scarpa's triangle*.

The abnormal group included 194 subjects with malignant lymphoma or leukemia. All diagnoses were confirmed by accepted standards, utilizing lymph node biopsy, blood studies, bone marrow aspiration, or autopsy. There were 78 (40.2 per cent) examples of "inguinal" adenopathy, which included adenopathy of all degrees and distribution in the groin (Table 68 and Fig. 214). This higher incidence (40.2 per cent) as compared with the normal controls (21.0 per cent) comes as no surprise. However, when this adenopathy was scrutinized as to distribution, it was found that 55 patients (28.4 per cent) had "triangle" adenopathy as compared with none in the normal con-

TABLE 68

DISEASE	TOTAL NUMBER OF CASES	INGUINAL ADENOPATHY		TRIANGLE ADENOPATHY	
		NO.	PER CENT	NO.	PER CENT
Normal controls	256	54	21.0	0	0
Lymphosarcoma	33	10	30.3	5	15.1
Leukosarcoma	12	7	58.3	5	41.6
Lymphocytic leukemia	57	36	63.1	29	52.6
Myelocytic leukemia	36	11	30.7	8	22.2
Acute stem cell leukemia	15	3	20.0	1	6.7
Monocytic leukemia	10	3	30.0	1	10.0
Hodgkin's disease	31	8	25.7	6	19.3
Totals for malignant disease	194	78	40.2	55	28.4
Infectious mononucleosis	24	9	37.5	3	12.5
Infection lower extremity	20	10	50.0	4	20.0

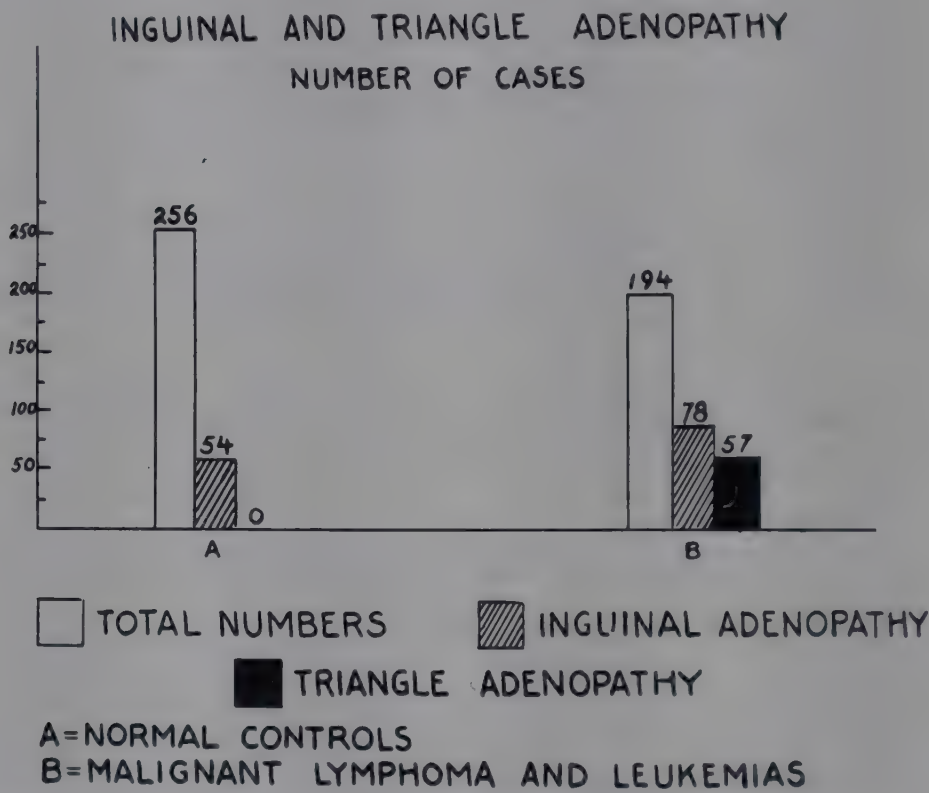


Fig. 214.—Inguinal and triangle adenopathy, normal controls and malignant lymphoma and leukemias. (Contributed by Dr. Harry Agress.)

trols. The degree of this "triangle" adenopathy was quite variable. In general, it paralleled the degree of "inguinal" enlargement, but occasionally it occurred in the absence of inguinal adenopathy or was more striking than the "inguinal" adenopathy. In two patients, "triangle" lymph node enlargement was the *only* adenopathy noted in the entire physical examination. Because we considered this adenopathy "significant," biopsy was performed, and diagnoses of malignant lymphoma were made in both instances. Generally, the degree of "triangle" adenopathy and "inguinal" adenopathy not only paralleled each other in incidence, but in various disease groups such adenopathy paralleled the occurrence of generalized adenopathy (see Table 68 and Fig. 215). For example, lymphocytic leukemia and leukosarcoma, which are usually associated with generalized lymph node enlargement, had the highest incidence of both "triangle" and "inguinal" adenopathy. On the other hand, in the acute "stem cell leukemias," where generalized adenopathy has a low incidence, both "triangle" and "inguinal" adenopathies

were minimal. Further analysis of these data failed to reveal any constant correlation between the incidence of groin adenopathy and the stage of the disease process. In general, the more advanced the disease the more striking was the adenopathy. Occasionally, "triangle" adenopathy was the initial presenting site of lymph node enlargement.

To test the validity of interpreting the significance of "triangle" adenopathy, a similar survey was made of nonmalignant diseases associated with lymphadenopathy. Twenty-four cases of acute infectious mononucleosis were studied; 9 cases (37.5 per cent) had "inguinal" adenopathy and 3 cases (12.5 per cent) showed "triangle" adenopathy (see Table 68 and Fig. 215).

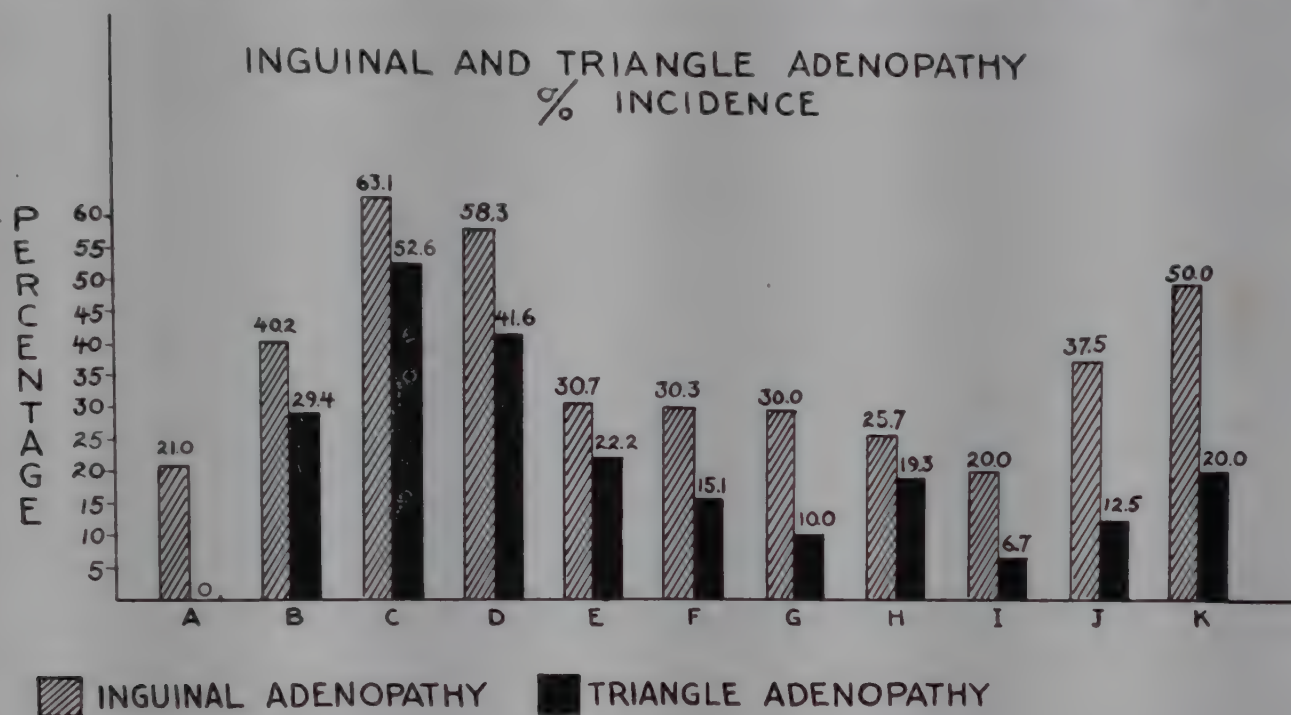


Fig. 215.—Inguinal and triangle adenopathy, percentage incidence. A, Normal control; B, malignant disease group; C, lymphocytic leukemia; D, leukosarcoma; E, myelocytic leukemia; F, lymphosarcoma; G, monocytic leukemia; H, Hodgkin's disease; I, acute stem cell leukemia; J, infectious mononucleosis; K, acute infection of lower extremity. (Contributed by Dr. Harry Agress.)

No other infectious diseases associated with generalized adenopathy were seen in sufficient numbers to be statistically valid. Twenty cases of infection of the lower extremity were examined and in 50 per cent of these, there was "inguinal" node involvement on the affected side only (see Table 68 and Fig. 215). Four cases (20 per cent) of infection of the lower extremity showed "triangle" adenopathy on the involved side. In all these instances of infection of a lower extremity, the disease process was quite obvious or a history of such infection was readily obtained. On the other hand, the cases of infectious mononucleosis sometimes posed a slightly more difficult problem. However, the clinical picture together with adequate blood studies generally resolved the problem of diagnosis.

From these observations, it is concluded that when adenopathy is present at the apex of Searpa's triangle, such lymph nodes are "significantly" pathologic. Palpable "triangle" nodes should arouse a high degree of suspicion of malignant lymphoma or leukemia. Acute infectious mononucleosis and infection of the lower extremity must be ruled out. On the contrary, the absence of "triangle" adenopathy does not militate against the diagnosis of

pathologic lymph node involvement in the groin. Other enlarged nodes may be significant and should be evaluated on such features as clinical picture, size, consistency, duration, and rate of growth.

Treatment of Leukemia and Malignant Lymphomata

There is as yet no satisfactory treatment of leukemia or the malignant lymphomata, but interest is great as the tremendous amount of basic research and animal experimentation in this field bear witness. Despite the generally poor results in treatment, advances have been made that suggest the possibility of success in the future. Perhaps the most outstanding feature of present-day therapy is the attack on these diseases with the use of chemotherapeutic agents based on some new concepts.¹ Basically, this concept is that the cancer cell (leukemic or otherwise) can be regarded in principle much like invading microorganisms. These latter can be destroyed by chemotherapeutic agents while in the human host. Then why not attempt to destroy cancer cells in the same way, without injuring normal cells? The basic principles of this approach are (a) the principle of "dynamic equilibrium," and (b) that of "specific nutrition." Principle (a) fundamentally states that all cells require a constant supply of proper nutriment to survive. Principle (b) states that different cell types may have different nutritional requirements either from their external environment or within their internal mechanisms. This concept has been referred to as "the heterogeneity of nucleic acid anabolism." The researches of the Sloan-Kettering group have been directed at applying these concepts by selective feeding of modified chemical agents to cancer cells effecting a starvation of the cell particularly directed at nucleic acid metabolism. This group has repeated in tissue culture of normal and cancer cells what can be shown in bacterial cultures, namely, selective destruction of cells by chemical agents.

SubbaRow's² synthesis of folic acid (pteroylglutamic acid) and the finding that the clinical course of acute leukemia was unfavorably affected by this substance or its conjugates led Farber and his associates³ to use folic acid antagonists in the treatment of acute leukemia in children. The Sloan-Kettering group has carried on extensive animal research with these chemical compounds, as well as others, with very promising results. Using 10 different compounds on 5- to 7-day-old tumors in mice, 1,275 animals (67 per cent) out of 1,910 treated were cured. In mouse leukemia, 44 per cent cures were effected with 6 selected compounds. Amethopterin, an analogue of Aminopterin, was found 98 per cent effective against Line I mouse leukemia but effected no cures in Line I/A, a resistant strain. However, when a combination of P-165 (azaserine or O-diazoacetyl-L serine) and 6-MP (6-mercaptopurine)* was used against this resistant Line I/A strain, 98 per cent of the

*Purinethol, Burroughs Wellcome Laboratories.

¹Progress Report, VII, New York, Sloan-Kettering Institute for Cancer Research, June, 1954.

²Angier, R. B., Boothe, J. G., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Holtquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M.: *Science* 102: 227, 1945. *Ibid.* 103: 667, 1946.

³Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F., Jr., and Wolff, J. A.: *New England J. Med.* 238: 787, 1948.

mice were cured of their leukemia. These results are most encouraging in that an approach appears possible that may shed a ray of hope in the treatment of human leukemias.

To date no human cures of leukemia by chemical or other agents have been effected. The literature on the use of these agents is voluminous and has been excellently reviewed by Wright and associates.⁴ Therapy with the folic acid antagonists has been the subject of a lengthy supplement of *Blood*.⁵ Chemotherapy of leukemia, Hodgkin's disease, and related diseases based on ten years' experience has been reported by Wintrobe and his group.⁶ The folic acid antagonists which have been used in the treatment of leukemia are Aminopterin, Amethopterin, Amino-an-fol, Adenopterin, and Aminoteropterin. All of these related compounds are pteroylglutamic or pteroylaspartic acid derivatives. Farber's enthusiasm for the results achieved with these antagonists has not been shared by everyone and results from their use have varied. Whatever else may be said about these compounds, one thing stands out in our minds: hopeful events have happened in patients with acute leukemia that never happened before these agents came along. Everyone who has treated acute leukemia, especially in children, agrees that the folic acid antagonists have a profound effect on this disease, which in the past left us dismayed and hopeless as it ran its speedy course unchanged by any known form of therapy. The most striking cases are those in which the peripheral blood picture may approach normal and in which the bone marrow shows considerably diminished leukemic activity. It is claimed that in some rare cases, autopsy failed to show any evidence of leukemia. Clinically, these patients display improved vigor, require fewer transfusions, and are generally useful. These remissions may last only a few days up to many months.

When one tries to analyze statistics as to the benefits derived from folic acid antagonist therapy, there are many variables that readily account for the differences in opinion. Cases of acute leukemia are fortunately relatively rare and very few observers have had an opportunity to manage sufficiently large enough numbers of patients to make their statistics entirely acceptable. The classification of acute leukemias may vary from one group to another, which adds another variable to statistical analyses. As with all investigations and therapeutic approaches to disease, the intensity of interest and enthusiasm of the investigator is often reflected in beneficial results in patients not achieved by physicians whose interest is more casual. This not only involves more careful dosage schedules but care in general management that adds some imponderables to successful therapy.

With folic acid antagonist therapy there is no unanimity of opinion as to whether treatment should be pushed to toxicity with high dosage and a rest period, or whether modest dosage schedules should be followed intermittently, or if there should be continuous therapy. Whatever schedule is followed, leukocyte counts and the blood picture are generally helpful and necessary in

⁴Wright, C. S., Sundharagati, B., Bass, J. A., and Bunner, A. E.: *Arch. Int. Med.* **92**: 357, 1953.

⁵Proceedings of the Second Conference on Folic Acid Antagonists in the Treatment of Leukemia, *Blood (supp.)* **7**: 97, 1952.

⁶Wintrobe, M. M., Cartwright, G. E., Fessas, P., Haut, A., and Altman, S. J.: *Ann. Int. Med.* **41**: 447, 1954.

following these cases. In some cases the white count may return to normal or near-normal values, with disappearance or diminution of leukemic cells in the blood spread.

One of the troublesome problems in many of these patients is that leukopenia is a common accompaniment of the natural history of acute leukemia and that the blood spreads may show relatively few leukemic cells. Studies of the bone marrow in such cases show a wildly active leukemic process. Should these patients be given a leukopenia-producing agent? If so, what should determine dosage? Situations such as these appear to require more courage than scientific knowledge. When toxicity occurs, the symptoms may be more distressing than those related to the leukemia: affection of the gastrointestinal tract, with gingivitis, stomatitis, anorexia, and bloody diarrhea. Folic acid and citrovorum factor, vitamin B₁₂, antibiotics, and liver preparations have been suggested to counteract those toxic effects but have been generally ineffectual. Hemorrhagic diatheses from thrombocytopenia or ulcerations in the gastrointestinal tract present serious problems.

Another complication of therapy is resistance to certain preparations in human beings just as in the mouse experiments previously cited. Such resistance does not militate against use of other agents.

Perhaps the results with the use of folic acid antagonists can be summarized by reiterating that in some cases effects occur that have not been previously observed in acute leukemia. These favorable responses occur much more frequently in children than in adults, and their duration is variable but with no expectancy of cure. Other forms of leukemia are not favorably affected by these agents.

6-MP (6-mercaptopurine, Purinethol) is a recent antimetabolite used in the treatment of leukemia. This substance acts as a purine antagonist against certain forms of bacteria, but its mode of action in leukemia is unknown, albeit apparently in a manner different from the folic acid antagonists. Burchenal and associates⁷ have summarized their clinical evaluation of this agent in a very comprehensive review including the pharmacology, clinical results, and toxicology together with a series of representative case histories. The results appear to follow those obtained with folic acid antagonists with more strikingly beneficial results in children with acute leukemia, 15 out of 45 such cases showing good clinical and hematologic remissions and another 10 cases showing partial improvement. In adults, remissions in acute leukemia have been only occasional, and temporary remissions have occurred in some cases of chronic myelocytic leukemia. The lymphomata, including lymphocytic leukemia and lymphosarcoma, were unaffected by 6-MP. In some instances of acute leukemia in children with resistance to folic acid antagonists or to ACTH or cortisone, 6-MP proved effective in producing some degree of remission. Resistance to 6-MP develops more rapidly than with the folic acid antagonists but there apparently is no cross-resistance between the two. Toxic effects were primarily hemopoietic with leukopenia and bone marrow depression. However, there is cessation of this process on withdrawal of the drug. Gastrointestinal symptoms were generally mild and not of the

⁷Burchenal, J. H., Murphy, M. L., Ellison, R. R., Sykes, M. P., Tan, I. C., Leone, L. A., Karnofsky, D. A., Craver, L. F., Daregon, H. W., and Rhoads, C. P.: *Blood* 8: 965, 1953.

order of severity of those produced by the folic acid antagonists. Tivey and Bross⁸ summarized the results of a conference on therapy with 6-MP as follows:

Of 337 children with acute leukemia treated in thirteen clinics, 36 per cent were improved. The percentage varied from 13 per cent in one clinic to almost 70 per cent in another. Variables such as dosage schedules, terminology, and the use of other agents in conjunction with 6-MP made interpretation of the results difficult.

In this same report, there is an illuminating block chart (Table 69) showing that as new compounds become available, more children with acute leukemia survive one year or longer with treatment. Ordinarily 5 per cent survive beyond one year; with Amethopterin and cortisone, approximately 30 per cent carry on for one year or longer; and with Amethopterin, cortisone, and 6-MP, 50 per cent survive the year.

Urethane (ethyl carbamate) has been found an effective and relatively simple agent in the therapy of chronic myelocytic leukemia and in multiple myeloma. As with all the other chemotherapeutic agents, a host of papers on the subject may be found in both the domestic and foreign literature. It effects a lowering of the leukocyte count in chronic myelocytic leukemia and at times may cause some diminution in organ enlargements in this disease. Its greatest recommendation is the ease of administration (oral) and its relative lack of discomfort. Toxic symptoms, other than those related to the hemopoietic system, are relatively mild and disappear upon withdrawal of the drug. Rare instances of severe liver damage may occur. Since leukopenia and thrombocytopenia of serious proportions may occur, treatment with urethane should be monitored with frequent observations of the blood picture. When the leukocyte count reaches normal levels, dosage should be diminished or lowered. Our impression is that urethane therapy holds a position in relation to the treatment of chronic myelocytic leukemia that arsenotherapy used to hold.

Nitrogen mustard (methyl-bis [β -chloroethyl] amine hydrochloride) is the mustard gas of World War I vintage and has had its rebirth as a chemotherapeutic agent in the treatment of the malignant lymphomata, being most successful in Hodgkin's disease. The nitrogen mustards are representative of a group of antileukemic agents categorized as radiomimetic. Although the mustards have been tried in many forms of leukemia, their effectiveness has been greatest in Hodgkin's disease, some cases of generalized lymphosarcoma, and perhaps have an occasional worthy application in other selected cases. The mustards are administered intravenously and may cause severe chemical phlebitis or severe reactions at the site of administration if accidentally infiltrated into the tissues. Nausea and vomiting of a disturbing degree are frequent toxic side reactions. There is a considerable difference of opinion as to just where to place these agents in relation to roentgen radiation therapy. Generally speaking, there are few if any beneficial effects produced by the mustards that have not been produced by roentgen radiation. Relative costs do not appear a basis for choice. Although nitrogen mustard is less expensive,

⁸Tivey, H., and Bross, I.: Quoted in Progress Report VII, New York, 1954, Sloan-Kettering Institute for Cancer Research, p. 14.

it is best handled under hospitalized conditions, adding to the cost of therapy. Toxic manifestations, such as nausea and vomiting, are much more frequent with mustards than with roentgen radiation. Toxic hematologic effects, particularly thrombocytopenia and leukopenia of severe degree, are more likely to occur after therapy with the mustards than with roentgen radiation. In general, where organ enlargements, such as localized lymphadenopathy or splenomegaly of considerable proportions, are present, direct radiation is much more likely to accomplish shrinkage of these organs than are the mustards. Pruritus, which may be very distressing in Hodgkin's disease, responds favorably more frequently to mustard therapy than to radiation therapy. It is frequently claimed that when the malignant lymphomata become resistant to roentgen radiation, the disease will still react favorably to the mustards and may become radiosensitive again. For this reason alternate courses of therapy with mustards and radiation have been recommended and in some investigations simultaneous therapy with both agents has been tried.

Triethylene melamine (TEM) was the first of a group ethylenimine derivatives having nitrogen mustardlike actions. Included in this group are triethylene phosphoramidate (TEPA), triethylene thiophosphoramidate (thio-TEPA), and diethylene phosphoramidate (DEPA). The therapeutic effects with these substances are fairly similar to those seen with the nitrogen mustards, Hodgkin's disease being the most favorably affected, although some control of chronic lymphocytic and myelocytic leukemia has been effected. An advantage over the mustard therapy is that these agents may be given orally. Among the toxic effects, the most significant are related to suppression of hemopoiesis, with leukopenia and thrombocytopenia not uncommon. Cumulative toxic effects may reach serious proportions, and since there is a narrow margin of safety between desired clinical effects and toxic reactions, careful monitoring of the hematologic status of these patients is essential.

Myleran (GT 41) is one of a series of sulphonic acid and other esters which were synthesized,⁹ particularly with the idea that they might exert nitrogen mustardlike biological action. Galton¹⁰ reported on the use of this agent in leukemia and found it effective in producing remissions in chronic myelocytic leukemia. Its major field of usefulness appears to be in this form of leukemia because of its depressive action on the myeloid series. Thrombocytopenia may be an important undesirable toxic effect.

Steroid therapy in the form of cortisone or corticotropin (ACTH) has been used in selected cases of leukemia and the malignant lymphomata. One gathers the general impression that these agents are most helpful in those cases which have as an associated phenomenon some secondary immunohematologic features, such as hemolytic anemia, thrombocytopenia, or leukopenia. Consequently, these agents have not been used as primary weapons against such malignant disease but rather as adjunctive therapy along with other therapeutic approaches. Such situations arise frequently in the acute leukemias and in selected cases of the chronic leukemias and malignant lymphomata in which splenic involvement is prominent. The incidence and character of toxic effects with ACTH and cortisone are similar to those ac-

⁹Haddow, A., and Timmis, G. J.: *Lancet* 1: 207, 1953.

¹⁰Galton, D. A. G.: *Lancet* 1: 208, 1953.

companying their use in other disease processes. These include the Cushing's syndrome (acne, hirsutism, edema, moon facies, hypertension, and glycosuria), mental depression, and psychoses.

Radiation therapy "is still the sheet anchor for treating chronic leukemias"¹¹ and, we might add, the treatment of malignant lymphomata. Radiation has been used in the form of external radiation or internal radiation (radioactive isotopes). Variations in dosage schedules and methods of application are numerous, with champions representing the various schools of thought. As with many other therapeutic procedures, the enthusiasm and persistence of the therapist color his views. The end results of radiation, regardless of method, appear to be about the same insofar as longevity and relief of symptoms is concerned. Toxic symptoms of external radiation include radiation sickness, which is seldom seen with isotope therapy. On the other hand, annoying hematologic complications, such as severe leukopenia, anemia, and thrombocytopenia, are more frequent with isotope therapy than with external radiation. The shrinkage of organ enlargements, such as splenomegaly or lymphadenopathy, can be more effectively accomplished with radiation therapy than with isotopes.

The most frequently used isotope is radioactive phosphorus (P^{32}). This agent may be administered orally or intravenously. There is a period of approximately 3 to 4 weeks before therapeutic response is seen. Colloidal gold (Au^{198}) has been used in some cases of chronic myelocytic leukemia¹² and it has been tried in the treatment of effusions caused by malignant neoplasms.¹³

Radiation therapy is not used in the acute leukemias. It has a very useful place in the malignant lymphomata, with external radiation generally favored over isotope therapy.

A host of miscellaneous forms of therapy has been reported with varying results. Myelokentric and lymphokentric acids¹⁴ have been administered on the basis of the reciprocal actions of substances affecting the myelopoietic and lymphopoietic systems. Vitamin B_{12} and other hematinics have been generally found ineffective. In addition, a large number of chemicals known to produce leukopenia in animals and human beings have been given limited therapeutic trials.

Transfusions play a very important role in the management of the anemia associated with leukemia and the malignant lymphomata. The ready availability of blood has been a major factor in the rehabilitation of many of these patients, whose anemia generally does not respond to oral hematinics unless there is an associated blood loss. It is our opinion that many of the benefits of modern care of these patients can be attributed to the free use of whole blood.

Almost all of the antibiotics have been used as adjunctive therapy in the treatment of the leukemias. The display of these agents has embodied both prophylactic therapy and the treatment of actual infection. As one reads

¹¹Wright, C. S., Mabry, D. S., Carr, R. D., and Perry, A. M.: *Arch. Int. Med.* **94**: 995, 1954.

¹²Andrews, G. A., and Tyor, M. P.: *J. Lab. & Clin. Med.* **42**: 777, 1953.

¹³Andrews, G. A., Root, S. W., Kerman, H. D., and Bigelow, R. R.: *Ann. Surg.* **137**: 375, 1953.

¹⁴Turner, D. L., Miller, F. R., and Flint, J. S.: *J. Nat. Cancer Inst.* **14**: 439, 1953.

some of the literature, there is an overtone suggesting that the antibiotics might have some beneficial influence on the leukemic process other than those effects related to infection. Our experience is that, in the absence of infection, the antibiotics can play only a questionable role.

TABLE 69.—CHEMOTHERAPEUTIC AGENTS USEFUL IN TREATMENT OF NEOPLASTIC DISEASES

AGENT	AVERAGE DOSE	DISEASES BENEFITED	EFFECT OF OVERDOSAGE; COMPLICATIONS
Urethane	0.5-1.0 gm.	Multiple myeloma Myeloid proliferative dis- eases Leukemia, chronic granulocytic Polycythemia vera?	Anorexia, nausea, vomiting, olfactory disturbance Leukopenia, pancytopenia (rare) Centrilobular hepatic degen- eration and necrosis
Nitrogen mustard (HN ₂)	0.1 mg. per kg. I.V. × 4-6	Lymphoid proliferative dis- eases Hodgkin's disease (generalized) Lymphomas, malignant Some anaplastic carcinomas	(Acute nausea, vomiting) Pancytopenia Marrow (temporary) de- pression
Triethylene Melamine (TEM)	2.5 mg., fasting with 2 gm. soda, per day × 2-3 initially; 1-5 mg. per 5-10 days as maintenance	Chronic lymphocytic leu- kemia Lymphocytic lymphoma Hodgkin's disease (general- ized) Chronic granulocytic leu- kemia Polycythemia vera Adenocarcinoma ovary	Anorexia, nausea, vomiting Leukopenia, severe Pancytopenia
ACTH	20 units I.V. for 15-18 hr. per day, or 20-40 units gel I.M. per day	Acute leukemia Chronic lymphocytic leu- kemia with marrow dam- age Hemopoietic malignancies with symptomatic hemo- lytic anemia	Edema Cushing's syndrome: moon facies, acne, hir- sutism, edema, hyper- tension, hyperglycemia (glycosuria) Hypokalemic, hypochloremic alkalosis Mental depression, psycho- ses
Cortisone	25-50 mg. q 4-6 hr.	Same as ACTH	Same as ACTH
Folic acid an- tagonists	Aminopterin, 0.5- 1.0 mg., orally, 1-3 × per week	Acute leukemia	Pancytopenia Mouth ulceration Diarrhea
6-Mercapto purine (Purinethol)*	1-2.5 mg. per kg., daily, initially	Acute leukemia Chronic granulocytic leu- kemia	Leukopenia Pancytopenia
Myleran*	6 mg. per day, orally, initially; 2-4 mg. per day maintenance	Chronic granulocytic leu- kemia	Leukopenia Pancytopenia

Revised from Rundles, R. W., Barton, W. B., and Coonrad, E. V.: South. M. J. 46: 259, 1953. By permission of authors and publisher.
*Personal communication, Wayne Rundles, M.D., Duke University.

With the growing consciousness of immunohematologic mechanisms, more and more cases of leukemia and lymphomata are being considered for splenec-
tomy. The patients with secondary hemolytic anemia are the ones in whom
splenectomy has been frequently considered, especially in cases of chronic
lymphocytic leukemia. Poor results with splenectomy have been recently
reported in 5 advanced cases of Hodgkin's disease.¹⁵ It would appear that

¹⁵Sykes, M. P., Karnofsky, D. A., McNeer, G. P., and Craver, L. F.: Blood 9: 824, 1954.

unless well-proved immunohematologic mechanisms can be demonstrated in an individual case, splenectomy should not be considered. Splenectomy, when effective, affects only the abnormal immunologic process, and whatever benefits accrue to the patient are the result of such influences. The basic disease is usually unaffected or the clinical course may be precipitated into a more rapid tempo.

In the choice of treatment of any patient, a host of factors must be given consideration. Undoubtedly, the philosophy of the therapist plays a leading role in such decisions. The utter pessimism of some physicians is understandable in the light of the death sentence imposed by a diagnosis such as leukemia or malignant lymphoma. The outlook has been brightened by the well-established effects of the antimetabolites in acute leukemia, where events have taken place that were never seen before. These results have modified the views of many in the direction of achieving "control" of these diseases in somewhat the same manner as diabetes is controlled. The approach to such control in our present state of knowledge is limited to a great extent by the untoward reactions of the agents used in combating these diseases. There are many times when the natural course of the disease is better left undisturbed than overzealously treated to the point where the discomfort from the therapy far outshadows that associated with the disease process. This implies a thorough knowledge of the natural history of the disease, which may be difficult to prophesy in individual cases but has been fairly well categorized in general. Many studies on life expectancy have been published; there is no conclusive proof that life expectancy is prolonged by our present-day treatment, except perhaps in acute leukemia, especially in children. The pessimistic school has raised the question of whether such increases in prolongation of life as may occur are even worth while and point to the added mental anguish and economic strain imposed upon patients and family as sufficient reason to question the wisdom of this prolongation. Social conscience and philosophy become so inextricably enmeshed in these discussions that at times we lose sight of our position. The immature press releases of the optimistic school are as much to be abhorred as is the nihilistic approach of their opposites. There is undoubtedly a middle ground of approach in which the comfort and usefulness of the patient should be our present target of therapy. Judicious application of present technics can accomplish these aims in many cases. In the meantime the genius that will effect real cure has not been revealed to us. The furious attack on nucleic acid metabolism may or may not be the proper answer but indicates an attitude of refusal to be stifled by traditional concepts. Along with this attitude comes a ray of hopefulness that satisfactory answers may come in the not too distant future. Whence will come the prophets carrying the glad tidings remains to be seen.

HEMOSTASIS AND ABNORMALITIES OF BLOOD COAGULATION

In the normal individual, blood courses through the vascular tree in a properly fluid state, carrying a multiplicity of substances and structures. Life itself is dependent upon the complex regulatory mechanisms that maintain an

exquisite balance of all the factors which combine to make for this state of normality. A basic requirement for sustaining such a status quo is an intact vascular system, which, while maintaining its integrity, still permits selective exchanges of vital materials between the circulatory system and the tissues. Although a great deal of knowledge has been accumulated concerning capillaries, there is perhaps more information concerning their reaction to injury and in disease than answers as to why they remain intact in the normal individual. Alterations in the character of the vascular bed may occur as the result of a multiplicity of causes, involving direct and indirect assault on the vessels themselves or the contents of the vessels. The end result of such attacks may be the escape of blood from the vessels, or the closure of vessels. It is quite apparent from these remarks that the study of any patient in whom the defense mechanisms for maintaining the normal liquid state of blood in an intact vascular system have been threatened involves consideration of many factors. These might be conveniently considered under the following general headings: (1) the state of the vascular tree; (2) the state of the coagulation mechanism; (3) the status of tissues and organs related to the elaboration of elements essential to maintaining normal coagulability of blood, notably the liver, spleen, and bone marrow.

Alterations in one or in combinations of these basic states may result in either a hemorrhagic diathesis or thrombosis. The clinical results of such derangements cover the entire spectrum from mild symptomatology to fatal outcome. In the study of patients with any such symptomatology, consideration of the causes involves complete history, physical examination, and laboratory examination. As in all clinical medicine, we cannot emphasize too strongly the need for thorough history taking and physical examinations *before* rushing to the laboratory for answers to such patients' problems. This orderly procedure will often circumscribe the disease process and eliminate the necessity of costly and time-consuming laboratory tests.

The history should completely consider the family history as well as the personal history of the patient. In the hemorrhagic disease group, hemophilia is undoubtedly the outstanding example. As knowledge of the factors involved in hemostasis has grown, many other examples of hereditary disease have been uncovered. Following is a list in which heredity plays a role:*

(1) Capillary purpura due to:

Hereditary capillary fragility

(pseudohemophilia, purpura of capillary weakness, von Willebrand's disease)

Hereditary capillary telangiectasia

(Rendu-Weber-Osler disease, Goldstein's heredofamilial angiomatosis, hereditary angiomata, hereditary hemorrhagic telangiectasia)

(2) Thrombocytopenic purpura due to:

Hereditary thrombocytopenia

Thrombocytopathic purpura

(Glanzmann's thrombasthenia)

*This list is based on the recommended nomenclature published by a committee sponsored by the American Medical Association and the American Society of Clinical Pathologists (Am. J. Clin. Path. 20: 575, 1959). The designations appearing in parentheses are synonymous terms found in the literature but which this committee recommends avoiding. Minor additions to the original list have been made by the author.

(3) Plasma defects affecting coagulation due to:

Unknown causes

(hereditary thrombasthenia, Glanzmann's thrombasthenia, constitutional thrombopathy, hereditary pseudohemophilia)

. Hypoprothrombinemia, familial

Fibrinogenopenia, hereditary

Deficiency of antihemophilic globulin (AHG)

Deficiency of plasma thromboplastin component (PTC)

Deficiency of plasma thromboplastin antecedent (PTA)

Deficiency of accelerator factor

(Factor V deficiency, labile factor deficiency, plasma Ac-globulin deficiency, parahemophilia)

In the personal history of the patient suffering from one of the hemorrhagic diatheses, many possibilities of acquired disease other than obvious trauma are to be considered. For the purpose of orientation as to the multiplicity of these factors, the following list has been compiled:*

(1) Capillary purpura, due to:

Mechanical cause

Infection

(Toxic purpura, Waterhouse-Friderichsen syndrome)

Emboli

Drugs

Allergy

(Schönlein's purpura, purpura abdominalis, Henoch's purpura, anaphylactoid purpura, purpura rheumatica, peliosis rheumatica, hemorrhagic capillary toxicosis)

Metabolic disturbances

Anoxia

Vitamin deficiency

(Scorbutic purpura, rutin deficiency)

Uremia

Senility

Endocrine

Neoplasms

Leukemia

Hemorrhagic sarcoma (Kaposi's sarcoma)

(2) Thrombocytopenic purpura, due to

(Morbus maculosis hemorrhagicus, Werlhof's disease, purpura hemorrhagica, primary or essential thrombocytopenia)

Idiopathic thrombocytopenic purpura

Thrombotic thrombocytopenia (Moschcowitz's syndrome)

Thrombocytic hypoplasia from endocrine disturbance or irradiation

Drugs

Allergy

Myelophthisis from unknown cause

Infection

Splenic disease

Congestive

Lipid histiocytosis

Sarcoidosis

Tuberculosis

Neoplasm, primary or secondary

*Loc. cit. preceding page.

Leukemia

Liver disease

Acquired antibodies

(3) Plasma defects affecting coagulation, due to

Hypoprothrombinemia

Vitamin K deficiency

Of newborn (melenia neonatorum, hemophilia neonatorum, morbus hemorrhagica neonatorum)

Deficient absorption from gastrointestinal tract

Obstructive jaundice

Steatorrhea

Drugs

Liver disease (pseudohemophilia hepatica)

Drugs, particularly Dicumarol ("sweet clover disease")

Fibrinogenopenia, due to liver disease

Endogenous anticoagulant

Drugs, especially heparin

Leukemia

Radiation

Venoms of snakes

Although this list appears formidable, an adequate history oriented along these lines may be very fruitful. Wearing of tight garters or other restraining garments may be the source of mechanical purpura. At times the investigation need go no farther than observation of such patients without this apparel. The complete disappearance of symptoms on this regime is generally sufficient proof of the causal factor. The list of drugs causing either capillary damage or thrombocytopenia is a very formidable one and every new therapeutic agent should be suspect. At times the incidence of purpura is so low as to make continuation of the drug generally permissible, withdrawing its use only in affected individuals. In other instances, purpura has been so universally associated with a drug as to completely bar its clinical usefulness.

Ackroyd¹ has exhaustively reviewed the **role of drugs**, as well as allergy and infections, in the causation of purpura, and we heartily recommend reading this scholarly paper on these related subjects. Among the drugs he lists are the following: Sedormid, arsenobenzol compounds, combined bismuth and arsenic therapy, sulfonamides, bismuth, quinine, quinidine, gold, iodine compounds, chrysarobin, phenobarbitone, Aleurate, digitoxin, ergot, colloidal silver, thiourea, dinitrophenol, menthol, DDT, cosmetics, and chloramphenicol. He emphasizes that such drug idiosyncrasy results in hypersensitivity due to repeated administration and questions reports of purpura occurring with the first dose of the drug. Once such hypersensitivity has developed it may persist for many years. In order to establish a cause-and-effect relationship between drugs and purpura, Ackroyd insists that administration of the drug be followed by the hemorrhagic state. Since this was not always done in case reports in the literature, there is some question concerning the role of some drugs as the causative agents in bleeding diatheses. Ackroyd calls attention to in vitro testing of alleged offending agents in addition to repetition of test

¹Ackroyd, J. F.: *Am. J. Med.* 14: 605, 1953.

doses in suspects. He has devised a series of such in vitro tests for detecting Sedormid hypersensitivity and suggests application of the same principles wherever drug sensitivity is suspect.

When the patient's platelet count has returned to normal, Sedormid is added to a blood sample, which is then observed for platelet agglutination and lysis. Both these phenomena occur in the blood of Sedormid-hypersensitive persons whereas addition of Sedormid to the blood of normal controls shows no change. If the Sedormid and control blood preparations are allowed to stand in tubes for several hours, permitting settling of the leukocytes and erythrocytes, the supernatant fluid in the two tubes is different. The supernatant layer in the control is opalescent and slightly opaque. On the other hand, the plasma layer of the Sedormid-sensitive blood sample is clear and transparent, which Ackroyd attributes to platelet agglutination and lysis. If a solution of Sedormid is added to the blood of a Sedormid-sensitive patient, clot retraction is poor, whereas no effect on clot retraction is noted in normal controls. The addition of Sedormid in saline to platelet-rich plasma of a Sedormid-sensitive patient causes lysis and agglutination of the platelets. The addition of saline alone to such platelet preparations has no effect on the platelets. The lytic effect on platelets appears to be associated with the fixation of complement, and complement is required to produce the effect. Platelet agglutination may be observed in the absence of complement.

From these observations, Ackroyd hypothesizes that "patients who have recovered from Sedormid purpura probably have in their blood stream an antiplatelet antibody which can cause lysis of platelets rendered immunologically antigenic by union with Sedormid." More recently, Steinkamp and co-workers² have extended this concept to in vivo experiments using quinine-sensitized systems as their drug milieu. The plasma of a quinine-sensitized patient was injected into a normal volunteer subject without effect on the platelets of the recipient. If, however, a dose of quinine were given to the same normal control just prior to the administration of the plasma, a prompt and sometimes dangerous thrombocytopenia ensued.

In drug sensitization of the types mentioned above, the usual mechanism of purpura production is thrombocytopenia. In some instances, a simultaneous lowering of leukocyte count or erythrocyte count may occur. Reports of bone marrow studies in the thrombopenic states due to drugs have been quite variable, and it is difficult to draw conclusions as to what constitutes changes characteristic of drug intoxication. We have seen morphologic changes in the megakaryocytes which are generally considered "toxic" and consist of abnormal and irregular staining of the cytoplasm with accompanying vacuolization. In some cases there is very little or no apparent cytolysis with platelet formation. In other cases, there are present many more youthful forms of megakaryocytes than in normal bone marrow, suggesting a "maturation arrest" phenomenon. The incidence of such increased youthful forms varies from case to case and from time to time in an individual case. Unless frequent serial bone marrow studies are performed, this phenomenon may be missed. In general, bone marrow studies are of relatively little assistance in either the diagnosis or the management of drug sensitivity thrombocytopenia. Bone marrow studies may be helpful in doubtful cases in excluding other causes of the thrombocytopenia such as leukemia or metastatic carcinoma.

²Steinkamp, R. C., Moore, C. V., and Doubeck, W.: *J. Lab. & Clin. Med.* 18: 18, 1955.

The vascular tree in drug sensitivity shows no significant histologic abnormality and is not as severely affected as are the platelets. However, in all cases of purpura due to drug sensitization, the capillary fragility test is positive. In some cases of Sedormid-sensitive patients without thrombocytopenia, a skin patch test with Sedormid may result in petechial hemorrhages over the site of application. Ackroyd hypothesizes an antigen-antibody relationship between Sedormid and the vascular endothelium in such cases.

To this group of drugs in which hypersensitivity plays a role should be added agents which apparently exert their influence more directly on the hematopoietic system. Among the commonly used preparations of this kind are the nitrogen mustards, Myleran, and 6-mercaptopurine, all of which may produce thrombocytopenia. In these instances, hypersensitivity does not appear to play a major role. Small doses administered to patients recovering from thrombopenia do not generally invoke a drop in platelets of an appreciable degree, and when there is a fall in the platelet count it is generally proportional to the dosage of the drug used. For these reasons, such agents may be repeatedly administered for the treatment of diseases of the hemopoietic system, whereas further display of offending drugs in drug-sensitive individuals is contraindicated.

The treatment of drug-induced thrombocytopenia is nonspecific. The most important step in the management of such patients is cessation of the offending agent and instruction of the patient against the future use of the drug. Since there is a possibility of cross-sensitivity by drugs of similar chemical structure, derivatives or close chemical allies of the original drug should be avoided or used very cautiously. If the purpura is severe enough to cause appreciable anemia, repeated transfusions may be of inestimable value during the acute phase. The efficacy of cortisone or ACTH is not well defined, but in the critically ill patient we would be inclined to administer either one or both of these hormones.

Perhaps one of the most difficult decisions in some patients is that concerning splenectomy. Although a few scattered reports appear in the literature, in which splenectomy was performed, it is our impression that no group has had a sufficient number of cases to warrant a strong stand favoring splenectomy, especially when one considers the apparently high mortality in the splenectomized group of drug-induced thrombocytopenias as compared with the "idiopathic" group. Our experience, which seems to coincide with others, is that the disease in drug-sensitive individuals is fairly self-limiting *if the offending agent is removed* and that the prognosis is good. Mild or moderate cases generally recover rather rapidly and splenectomy should not even be considered for this group. Even in the severe group, splenectomy should be considered only with the gravest reservations. We have observed long-standing severe thrombocytopenic purpura in three patients treated with "depot" preparations of gold salts for rheumatoid arthritis. These patients presented the special problem of the noxious agents remaining active within their body and there was no way of removing the offender quickly. Despite their grave condition, they were treated with multiple transfusions and ultimately re-

covered. BAL (British anti-lewisite) was used as an adjunctive therapy since we were dealing with a heavy metal intoxication. The effect of BAL in these cases was questionable.

Ackroyd admirably and thoroughly presents the picture of the **Henoch-Schönlein syndrome**, pointing out that in the vast majority of cases the etiology is unknown. Although allergy is invoked as a basic mechanism of this syndrome, relatively few cases have been proved as allergies. Ackroyd lists a small proportion of cases gleaned from the literature in which foodstuffs have been implicated. Included in this list are nuts, anchovy paste, blackberries, milk, eggs, potatoes, wheat, chicken, beans, plums, pork, onions, strawberries, fish, lamb, tomato, chocolate, popcorn, rolled oats, carrots, pineapple, apple, orange, and string beans. Milk, eggs, chocolate, beans, and wheat have been the most common offenders. It is to be emphasized that these cases constitute only a small minority of the total number of Henoch-Schönlein syndrome. The purpuric lesion in this syndrome is always associated with an exanthematous lesion, whereas in other purpuras the surrounding skin is normal. Originally, this symptom complex was divided into three groups based chiefly on the predominance of symptoms; namely, (1) the purpuric group ("purpura simplex"), (2) skin and joint group ("Schönlein's purpura" or "purpura rheumatica"), and (3) skin and gastrointestinal group ("Henoch's purpura" or "purpura abdominalis"). These groupings have been found arbitrary with mixtures of the symptoms varying in degree from case to case, suggesting a syndrome rather than fixed disease entities. At the onset the skin lesions may be simple purpura or may initially begin as erythematous papules, the centers of which become purpuric. It is this purpuric aspect that may suggest a blood dyscrasia. However, coagulation studies, including platelet counts, clotting time, bleeding time, and clot retraction, are normal. Capillary fragility may be increased in some cases. These normal blood findings, coupled with clinical features of acute joint or abdominal symptoms, should serve readily to differentiate the Henoch-Schönlein syndrome from a true blood dyscrasia. Treatment for this symptom-complex is generally nonspecific. If specific foods can be implicated, these should be eliminated from the diet. ACTH therapy has been used with questionable results. As a rule, the disease is self-limiting with a good prognosis, nephritis being the most serious complication. In the visceral group, abdominal pain may be so severe as to result in unnecessary surgery. However, in rare cases submucosal hemorrhage into the intestinal tract may be of sufficient degree to cause intestinal obstruction, perforating hemorrhagic ulcers, or intussusception.

Infection may be attended by purpura. Ackroyd divides these cases into the following groups: (1) Those associated with a Henoch-Schönlein syndrome. (2) Septicemias, in which purpura is a common feature of the disease process. (3) Hemorrhagic forms of acute infections, such as secondary streptococcus septicemia. (4) Purpura fulminans, occurring chiefly in children, in which the skin only is involved by purpura and a rapidly fatal course is the rule. Fulminating infections with adrenal involvement constitute the Waterhouse-Friderichsen syndrome. (5) Miscellaneous infections in which purpura is a rare complication. Although scarlet fever is the disease in which purpura occurs most commonly, the incidence of purpura is low (0.02 to 0.03

per cent). Other infections listed are acute infectious mononucleosis, varicella, rubella, diphtheria, measles, vaccinia, infectious hepatitis, tuberculosis, malaria, brucellosis, acute mastoiditis, and acute upper respiratory infections. In most of these infections, blood studies are normal other than those changes characteristic of the infection. In some cases thrombocytopenia may occur. When splenomegaly and thrombocytopenia occur simultaneously in a disease such as acute infectious mononucleosis, the clinical picture may simulate leukemia so closely as to cause concern. Careful examination of the blood spread revealing the typical changes in the lymphocytes of infectious mononucleosis should readily distinguish the disease from leukemia. Increased capillary fragility may be present more frequently than thrombocytopenia.

To Ackroyd's list of infections noted above should be added such diseases as typhus, typhoid fever, meningococcus meningitis, and subacute bacterial endocarditis. In some of these the petechial lesions appear to be the result of vascular thrombi or emboli rather than increased capillary fragility.

The acquired metabolic disturbances associated with increased capillary fragility listed form a **miscellaneous group of purpuras** of relatively infrequent occurrence. Vitamin C deficiency results in changes in the capillary endothelium cement substance, making vessels excessively permeable. The mechanism of senile purpura is generally related to the lack of supporting structure of the skin. The purpura of nephritis is poorly understood and generally attributed to changes in capillary permeability. Of the endocrine disturbances, Cushing's syndrome has been mentioned. "David's" disease¹ has been etiologically attributed to abnormal ovarian function and is usually athrombopenic. Minot² has described recurrent purpura associated with the menstrual cycle. Injection of large amounts of estrogen into animals has resulted in thrombocytopenia; Watson³ has reported this occurrence in males receiving large doses of estrogenic hormone for prostatic carcinoma. This association of purpura and estrogen therapy is not a common occurrence in the administration of estrogens at the dosage levels used in routine therapy of the menopause.

Insofar as the history is concerned, a group of hemorrhagic disorders that may be uncovered relates to those exposed to **irradiation**. Until recent years, our concern in human beings was limited to chronic exposure to x-ray with inadequate protection. The individuals most frequently involved were professional personnel, such as physicians and technicians. Rarely, patients have been sufficiently overexposed to cause hemopoietic disturbances. Industrial exposure to radium, such as those engaged in watch-dial painting with radioactive substances, formed an interesting and unusual chapter in radiation exposure. In most of these instances, the hemopoietic disturbance was slow in appearing and had the picture of an aplastic anemia. Thrombocytopenia was only one facet of the hematologic disturbance generally attributable to bone marrow destruction or hypoplasia. Chronic exposure to radiation has been a relatively insignificant cause of thrombocytopenic purpura in clinical experiences in recent years. Any complacency that we may have developed in recent years concerning irradiation protection has blown sky high with the

¹David, W.: *Med. Klin.* 22: 1755, 1926.

²Minot, G. R.: *Am. J. M. Sc.* 192: 445, 1936.

³Watson, C. J.: *J. Lab. & Clin. Med.* 32: 606, 1947.

atomic bombs dropped on Japan in World War II. A vast literature concerning acute radiation effects involving total body radiation has been accumulated both experimentally and clinically. The experimental data have been more carefully controlled in the reports of Cronkite and Brecher,⁴ Allen,⁵ and Tocantins.⁶ In the discussions of these authors at the Fifth Conference on Blood Clotting and Allied Problems, the general conclusion was that bleeding diatheses accompany acute total body radiation, but are not necessarily the major cause of mortality in exposure to high magnitude acute radiation. When a hemorrhagic syndrome does occur, it is not altogether limited to thrombocytopenia and effects on the bone marrow. There occur widespread disturbances of the bone marrow. There also occur widespread disturbances of the total mechanism of hemostasis involving both the vascular tree and various complexes of the clotting mechanism.

The introduction of therapeutic radioactive isotopes has been accompanied by a new radiation hazard. This not only involves professional personnel but patients as well. From a hematologic standpoint, the personnel working with **radioactive isotopes** are exposed to the dangers of chronic radiation effect of the magnitude related to work with x-ray. Patients receiving therapeutic doses of such isotopes, on the other hand, are subject to more acute exposure schedules and the incidence of thrombocytopenia is sufficiently great to warrant consideration of this complication before embarking on such therapy. The clinical dosages of radioactive phosphorus in the treatment of polycythemia vera and the leukemias are in the range of thrombopenia production. Generally, the thrombocytopenia appears from 4 to 5 weeks after administration and is symptomatically mild. However, bleeding may reach sufficient proportions for transfusion therapy. In most instances, the platelet count returns to normal after a period of weeks but may persist for months. In some instances of leukemia it is impossible to decide whether persistent thrombocytopenia is a complication of therapy or of the disease. At times one may be faced with the decision of treating the leukemic patient who already has thrombocytopenia with radioactive isotope. The resolution of this dilemma is generally based more on philosophy than science. Radioactive gold salts in therapeutic dosages may also result in thrombopenic states. Radioactive iodine in the usual therapeutic dose for the treatment of hyperthyroidism is not accompanied by a hematologic change. Tracer doses of many tagged substances, such as iron, cobalt, iodine, chromium, phosphorus, and iodine, are not attended by significant changes in the vascular tree or hematopoietic system.

In the **personal history** of any patient with a hemorrhagic diathesis, questioning relative to the gastrointestinal tract, biliary system, and the liver is important. Chronic diarrhea, such as occurs in sprue, may result in poor absorption of **vitamin K**. A history of jaundice with clay-colored stools should suggest obstruction of the biliary tract with resultant vitamin K deficiency. Liver disease may be difficult to elicit in the history but history of exposure to hepatotoxic substances may be helpful, as, for example, **carbon tetrachloride** acci-

⁴Cronkite, E. P., and Brecher, G.: *Blood Clotting and Allied Disorders*, New York, 1952, Josiah Macy, Jr. Foundation, pp. 171-212.

⁵Allen, J. G.: *Ibid.*, pp. 213-246.

⁶Tocantins, L. M.: *Ibid.*, pp. 247-279.

dentally inhaled in several ways. We have seen cases resulting from the escape of this agent from fire extinguishers. In one instance, the patient used a fire extinguisher in a closed basement. In another instance, the driver of an Army vehicle was intoxicated by carbon tetrachloride escaping from an extinguisher under the driver's seat of his car. Carbon tetrachloride poisoning has been observed in persons engaged in the cleaning of clothes. Phosphorus and chloroform poisoning may result in acute hepatitis with attendant liver damage.

Acute liver disease, in the form of **virus hepatitis** or **homologous serum jaundice**, may follow injections with improperly sterilized needles or syringes, transfusions of whole blood or plasma, or the administration of yellow fever vaccine. It is well to remember that the latent period between the injection and the development of acute liver disease may be several months. In all these instances of acute hepatocellular damage, capillary fragility may be increased; platelets are generally normal but other elements necessary for coagulation are diminished (usually plasma components).*

Physical examination of the patient with hemorrhagic disease should be thorough. Examination of the skin for purpura or petechiae should be performed in a good light. Purpura occurring at sites not subject to trauma should arouse suspicion of a hemorrhagic tendency. Petechiae arranged in linear fashion suggest mechanical origins, such as tight belts, brassières, corsets, girdles, arm bands, stocking garters. Such linear arrangement should also suggest self-inflicted mechanical trauma. The occurrence of numerous telangiectasia suggests the possibility of a hereditary form of capillary fragility. Attention is called to possible bleeding from such telangiectases in the gastrointestinal tract. In such instances, skin telangiectases may suggest this possibility.

The mucous membranes are often the sight of petechial hemorrhages and include any mucous membrane that can be visualized. Good lighting is important in this search. In Negroes, the mucous membranes may be the only visible site of abnormal bleeding. The conjunctivae of the eyes and the buccal mucosa are readily visible. Nasal examination for petechiae should be performed with the aid of a nasal speculum and a head mirror. Proctoscopy and vaginal speculum examinations may reveal mucous membrane petechiae. Funduscopic examination should be included in a search for petechiae.

Jaundice of the skin, sclerae, or mucous membrane should direct attention to the liver and biliary system.

Examination of the **gums** revealing marked thickening, pallor, and ready bleeding on manipulation is suggestive of leukemic disease.

Adenopathy of general distribution arouses the suspicion of malignant lymphoma or leukemia. In this connection it has been our experience that inguinal lymphadenopathy extending to the apex of Scarpa's triangle is particularly significant. This "triangle adenopathy," as we have come to call it, is a helpful diagnostic sign when recent regional infection is ruled out. Its presence bilaterally should always arouse the suspicion of malignant lymphoma.

*Refer to pages 565, 1185, and 1725 for further information on the transmittance of viral hepatitis.

Splenomegaly will direct attention to this organ system as the reflection of possible blood dyscrasia. However, the absence of splenic enlargement should not be a deterrent in pursuing such a diagnosis. Many cases of thrombocytopenic purpura display no splenomegaly. The diseases associated with hereditary plasma defects of coagulation, such as hemophilia, fail to show organ enlargement. Splenic enlargement is not particularly striking in affections such as tuberculosis and sarcoidosis. The spleen may be fairly large in such primary neoplasms as lymphosarcoma. In the chronic leukemias such as chronic myelocytic and chronic lymphocytic leukemia, splenomegaly is usually prominent and may reach tremendous proportions. However, in the acute leukemias, where thrombopenia is generally more striking than in the chronic leukemias, splenomegaly may be either absent or negligible.

Hepatomegaly may reflect primary liver disease, such as hepatitis, cirrhosis, or carcinoma. On the other hand, hepatomegaly may also reflect systemic disease, such as leukemia. In general, chronic leukemia is associated with a higher incidence and greater degree of hepatomegaly than acute leukemic disease.

A combination of hepatosplenomegaly suggests chronic leukemia, as well as portal hypertension syndromes. In this regard, hepatosplenomegaly should suggest the group of "hypersplenisms" in which thrombocytopenia often is a feature.

Cardiac examination revealing murmurs may suggest the possibility of bacterial endocarditis.

The presence of **acute joint involvement** may suggest the Henoch-Schönlein syndrome or acute rheumatic fever. Chronic joint involvement may be remindful of lupus erythematosus disseminata, which has been attended by purpura at times.

Abnormal neurological findings will parallel the site and extent of central nervous system hemorrhage. We have observed subarachnoid hemorrhage as the only presenting clinical picture in a case of thrombocytopenic purpura.

On rectal examination, the finding of tarry stool on the examining glove is indicative of **gastrointestinal bleeding**. This blood loss may or may not be associated with a hemorrhagic diathesis.

HEMORRHAGIC DIATHESES

Purpura

Purpura is characterized by hemorrhagic discoloration under the skin without apparent reason. These discolorations may be small, when they are called "petechiae," or large, when they are called "ecchymoses" or "vibices." These spots may appear temporarily, at intervals, or as a chronic condition. We have a simple condition called "purpura simplex" or a very serious condition called "purpura fulminans." The severe cases are usually associated with spontaneous or easily induced uncontrollable internal or external hemorrhages, and the condition is known as "purpura hemorrhagica."

Purpuric cases may be separated hematologically and clinically into two groups: one, which bears a close relationship to all kinds of infectious con-

TABLE 70.—THE HEMORRHAGIC DISEASES*

TERM TO BE USED	TERMS TO BE AVOIDED
<i>Capillary purpura</i> , due to	Symptomatic purpura, nonthrombopenic purpura, purpura simplex, purpura fulminans, erythema simplex
Unknown cause	
Mechanical cause	
Infection	
Emboli	
Drugs	
Allergy	
	Toxic purpura, Waterhouse-Friderichsen syndrome
	Schönlein's purpura, purpura abdominalis, Henoch's purpura, and anaphylactoid purpura, purpura rheumatica, peliosis rheumatica, hemorrhagic capillary toxicosis
Metabolic disturbance	
Anoxia	
Vitamin deficiency	
Uremia	
Senility	
Endocrine	
Hereditary capillary fragility	
Hereditary capillary telangiectasia	
Neoplasms	
Leukemia	
Hemorrhagic sarcoma	
<i>Thrombocytopenic purpura</i> , due to	Scorbutic purpura
Unknown cause	
Idiopathic thrombocytopenic purpura	Purpura cachectica
Hereditary thrombocytopenia	Pseudohemophilia, purpura of capillary weakness, von Willebrand's disease
Congenital thrombocytopenia	Rendu-Weber-Osler disease, Goldstein's hereditary familial angiomatosis, hereditary angiomata, hereditary hemorrhagic telangiectasia
Thrombotic thrombocytopenia	
Thrombocytic hypoplasia, from	Kaposi's sarcoma
Endocrine disturbance	Morbus maculosis hemorrhagicus, Werlhof's disease, purpura hemorrhagica, primary or essential thrombocytopenia
Irradiation	Hemogenia, hemogenic syndrome, idiopathic thrombocytopenia
Drugs	
Allergy	
Myelophthisis, from	
Unknown cause	
Infection	
Splenic disease	
Congestive	
Lipid histiocytosis	
Sarcoidosis	
Leukemia	
<i>Thrombocytopathic purpura</i>	
<i>Thrombocytosis</i> , due to	Moschkowitz's syndrome
Unknown cause	
Idiopathic thrombocythemia	
Polycythemia vera	
Neoplasm	
Leukemia	
Splenectomy	
<i>Plasma defects affecting coagulation</i> , due to	Glanzmann's thrombasthenia
Unknown cause	Thrombocythemia
Hypoprothrombinemia	
Unknown cause	
Vitamin K deficiency	
Of newborn	
	Hereditary thrombasthenia, Glanzmann's thrombasthenia, constitutional thrombopathy, hereditary pseudohemophilia
	Hemorrhagia universalis
	Melena neonatorum, hemophilia neonatorum, morbus hemorrhagica neonatorum

*Reproduced from the Am. J. Clin. Path. 20: 574-579, 1950, by courtesy of the Editor and of the Williams and Wilkins Company, Baltimore.

TABLE 70—CONT'D

TERM TO BE USED	TERMS TO BE AVOIDED
Deficient absorption Obstructive jaundice Steatorrhea Drugs Liver disease Familial Drugs Dicumarol Fibrinogenopenia, due to Unknown cause Liver disease Heredity Deficiency of accelerator factors, due to Familial Hemophilia Hereditary Sporadic Endogenous anticoagulant Unknown cause Drugs Leukemia Radiation	Pseudohemophilia hepatica "Sweet clover disease" Hypofibrinogenemia, pseudohemophilia, afibrinogen- emia Pseudohemophilia hepatica Factor V deficiency, labile factor deficiency Parahemophilia

TABLE 71.—SUMMARY OF DIAGNOSTIC FACTS IN PURPURIC DISEASES

NATURE	ATHROMBOPENIC PUR- PURA	THROMBOPENIC PURPURA (WERLHOF)	HEMOPHILIA
	VASCULAR INJURY	LACK OF BLOOD PLATE- LETS	DISTURBED COAGULATION
Etiology	Infections, anaphylactoid conditions (serum dis- ease, rheumatic condi- tions, etc.)	According to Frank "es- sential," or constitu- tional reactions in la- tent infections, etc., also associated with leucemias, osteosclero- sis, endocrine disor- ders, etc.	Congenital, confined al- most exclusively to men, transmitted by apparently healthy women, aggravated by familial tendency (families subject to hemophilia).
Blood find- ings	Coagulation normal, sometimes shortened. Normal or somewhat extended time of bleed- ing. Blood platelets normal, increased, or slightly decreased. Slightly infectious leu- kocytic findings. Later anemia.	Coagulation normal, re- tractability absent, time of bleeding much prolonged. Blood platelets much de- creased or absent. Sometimes infectious leukocytic findings. After unfavorable course, severe aplastic anemia.	Coagulation prolonged in various degrees. Time of bleeding often de- creased. Blood plate- lets normal. Leuko- cytes normal, or lymphocytosis.
Clinical	Purpura-exanthemata, hemorrhages from gums, tissue hemor- rhages. Least harm- ful course, seldom ag- onal.	Purpura-exanthemata (often greatly ex- tended) hemorrhages of mucous membrane, intestinal hemorrhages, etc., acute or chronic, benign or foudroyant- malignant course. Fre- quently splenic tumors.	Sudden hemorrhages from small or internal wounds. Blood effu- sions into the joint. Periodically increased. Often fatal hemor- rhages from slightest cause.
Therapy	Directed, if possible, against etiology; oth- erwise symptomatic. Cortisone and ACTH	Symptomatic, blood transfusion, or roent- gen ray treatment of spleen. Finally sple- nectomy. Vitamins are recommended. Cortisone and ACTH (with modifications)	Blood, fresh plasma, or antihemophilic globu- lin.

Schilling: "The Blood Picture" (with modifications).

ditions, or which has an infectious character, with fever, erythemas, edema, intestinal conditions, nephritis, and neuritis. This has been called "anaphylactoid purpura" of Glanzmann, with many clinical subvarieties. The other type is idiopathic without fever and is known as "morbus maculosus werlhofi." It may be acute periodic, or chronic, or it may be a symptomatic accompaniment of a severe blood disease.

Another classification of the hemorrhagic states based on the familial hemorrhagic tendency, the physical signs, and the abnormalities of the blood has been suggested by Mettier.*

It is to be noted that the actual cause of platelet deficiency in a given patient may be undetermined. Therefore, these cases are called idiopathic or essential thrombocytopenia. Certain investigators believe that the spleen is responsible for the platelet deficiency by actually destroying these elements of the blood,¹ by removing them by phagocytosis, or through some regulatory influence exerted on the megakaryocytes in the bone marrow.† Others‡ believe that severe bleeding from the mucous membranes may be associated with a deficiency of ovarian hormone and concomitantly with a reduction in the circulating platelets. See section on Immunohematology for further comments, pages 539 ff.

The Werlhof group shows a marked decrease in blood platelets, or the "essential thrombopenia" of E. Frank. Forty thousand blood platelets are estimated as the clinical limit of the hemorrhages; purpuric bleedings may appear below this number, for the reason that the mechanical element resident in the thromboplastic platelets and the consequent retractability of the coagulum are not present to close the wound. Vascular injury, however, is considered essential for the existence of this condition.

The bone marrow in essential purpura has been studied with the end in view of finding the cause of this condition in that organ. There are two theories regarding this: one by Frank,² who claims that the fault is in the bone marrow; one by Kaznelson,³ that it lies elsewhere. Lawrence and Knutti⁴ reported bone marrow studies on a number of these cases and found that in two cases there were diminished numbers of megakaryocytes in the bone marrow; in other cases they found a perfectly normal bone marrow. These results indicate that there are probably two types of this disease, one due to bone marrow disturbance, the other due to some other condition.

Purpura is related to the hemorrhagic diatheses found in avitaminoses, infectious and toxic-vascular conditions, and serum disease, and it is also related to constitutional conditions. It appears to have some relationship to real hemophilia which is a familial constitutional condition, seen especially in men, but transmitted by apparently healthy mothers.

*Mettier, Stacy R.: J. A. M. A. 108: 83, 1937.

†Krumbhaar, E. B.: *Physiol. Rev.* 6: 160, 1926.

‡David, W.: *Med. Klin.* 22: 1755, 1926.

Nagy, G.: *Ztschr. f. klin. Med.* 102: 284, 1925.

¹Brill, N. E., and Rosenthal, Nathan: *Arch. Int. Med.* 32: 939, 1923.

²Frank, E.: *Berl. klin. Wchnschr.* 52: 490, 1915.

³Kaznelson, P.: *Wien. Klin. Wchnschr.* 29: 1451, 1916.

⁴Lawrence, J. S., and Knutti, R. E.: *Am. J. M. Sc.* 188: 37-41, 1934.

The Megakaryocytes in Idiopathic Thrombocytopenic Purpura, a Form of Hypersplenism

Dameshek and Miller⁴ reported their results in a study of idiopathic thrombocytopenic purpura in special relationship to the megakaryocytes.

Method.—

The peripheral platelet count was made by the indirect or wet film technic of Dameshek,⁵ using an isotonic aqueous solution of sodium citrate containing brilliant cresyl blue. The normal platelet count by this method ranges from 400,000 to 900,000 per cubic millimeter, with an average normal count of about 600,000 per cubic millimeter.

Sternal puncture was performed in these cases by the introduction of a simple sternal puncture needle through the anterior lamella and into the marrow space, at a point on the sternum between the third and fourth intercostal spaces. Drops of aspirated material were immediately placed, without the further use of anticoagulant or other material, on carefully cleansed new glass slides and gently spread with minimum pressure by means of another slide. The preparations were allowed to dry in the air and were stained first with Wright stain, then with Giemsa stain, following which coverslips mounted in balsam were affixed.

For making the megakaryocyte counts, a rectangle was cut out and placed over the slide to be studied. The megakaryocytes in this area, containing approximately 20,000 oil immersion fields, were counted and expressed in terms of a million nucleated cells. At least one-half million nucleated red cells were counted, the count being facilitated by first accurately enumerating the nucleated red cells in 20 oil immersion fields.

The differential count of the megakaryocytes can be easy or quite difficult. The typical huge adult forms are readily defined, but recognition of their precursors requires much patience and study. The predominant mode of origin of the megakaryocyte is probably from a stem cell or megakaryoblast, which in turn probably originates from the pluripotential histiocyte or hemohistioblast.

For a description of the various types of megakaryocytes, refer to pages 645 and 646.

Dameshek and Miller concluded as follows regarding megakaryocytes in thrombocytopenic purpura:

The megakaryocytes of the sternal bone marrow at biopsy were studied in 11 cases of idiopathic thrombocytopenic purpura and compared with those of 10 normal cases, 5 of thrombocytopenic purpura associated with various types of splenomegaly, and of a large group of miscellaneous hematologic conditions, including leukemia, associated with a reduction in platelets.

Megakaryocyte counts expressed in terms of a million nucleated red cells and differential counts of megakaryocytes were performed. The megakaryocytes were classified as megakaryoblasts, promegakaryocytes, and mature forms, and were further subdivided into those showing granularity, platelet production, degenerated forms, and mitoses.

⁴Dameshek, W., and Miller, E. B.: *Blood* 1: 1, 27-49, Jan., 1946.

⁵Dameshek, W.: *Arch. Int. Med.* 50: 579-89, 1932.

In the *normal cases*, not more than 300 megakaryocytes per million nucleated red cells were present, and an average of 68.6 per cent showed platelet production.

In *acute idiopathic thrombocytopenic purpura*, although the platelets in the circulating blood were rare, megakaryocytes were increased, being present in a proportion of 366 to 743 per million nucleated red cells. Platelet production was, however, greatly diminished and found in only 8 to 19 per cent of all megakaryocytes. Following splenectomy, there was a striking increase in platelet production, which was now present in 69 to 85 per cent of all cells; the large masses of new platelets in the marrow were often very striking.

In *chronic idiopathic thrombocytopenic purpura*, the megakaryocytes were considerably increased over normal values, but showed great diminution in platelet production; following splenectomy, extreme degrees of platelet production from megakaryocytes took place.

In *splenomegaly of nonleukemic origin* (cirrhosis, splenic vein thrombosis, Gaucher's disease, Felty's syndrome), the megakaryocytes were somewhat increased, but platelet production was normal.

In *aplastic anemia, lymphosarcoma, acute leukemia*, and other diseases invading or destroying the bone marrow, the megakaryocytes were conspicuously reduced, the few remaining cells present being of normal morphology.

Finding of increased megakaryocytes and greatly diminished platelet production in the marrow before splenectomy and the striking increase in platelet production after splenectomy indicate a definite pathogenetic relationship of the spleen to the disease. Idiopathic thrombocytopenic purpura is probably a form of hypersplenism (splenic thrombopenia) in which, through a possible hormonal mechanism, the megakaryocytes of the bone marrow are inhibited from normal platelet production and delivery.

The marrow findings in idiopathic thrombocytopenic purpura are sufficiently characteristic to be of diagnostic value in differentiating the disease from leukemia and other conditions associated with a low blood platelet count.

Hemophilia

Not too many years ago, most of us had the impression that we fairly well knew the classical disease, hemophilia, as an entity occurring only in males and transmitted through females as a recessive sex-linked Mendelian characteristic. The abnormal bleeding tendency was known from the early days of the Hebrews who recognized its occurrence after circumcisions, and more recent history has been enlivened by the occurrence of hemophilia in the royal families of Europe. Within medical circles, the abnormally prolonged clotting time of hemophilic blood was well known and the therapeutic value of blood transfusions well established.

Any complacency about our knowledge of hemophilia has been fervently shaken by the coagulationists. This state of affairs has come about chiefly through recent advances in knowledge of the factors involved in blood coagulation. Hemophilia is no longer a simple disease and there is some ques-

tion, even in the minds of experts, as to just what the disease is. Dameshek¹ has collected the views of outstanding experts in the field in an attempt to answer this question.^{2, 3, 4, 5, 6, 7, 8, 9, 10}

As might be expected, there are the same differences of opinion about defining hemophilia as there are concerning nomenclature and theories of blood coagulation. The center of the controversy is the nature of the plasma and platelet factors, excluding calcium, which take part in the conversion of prothrombin to thrombin. A brief glance at the theories of blood coagulation in this area readily reveals many, but not all, of the differences. We heartily recommend reading this symposium of Dameshek as an enlightening (or perplexing) presentation of how utterly stirred up the concept of hemophilia has become.

Whereas in times past, our clinical concern about hemophiliacs was limited to males, we now must consider both male and female bleeders as possible hemophiliacs. Hereditary patterns are being found in the various newly described forms of hemophilia or hemophilia-like diseases as was true of the classical disease. However, acquired defects of clot-promoting factors have been recognized in syndromes simulating hemophilia.

The laboratory diagnosis of hemophilia traditionally rested on a prolonged clotting time. This is no longer true. Although most of the hemophilia-like diseases may have a prolonged clotting time, many instances with a normal coagulation time are being uncovered. Today, in the study of any patient with a bleeding diathesis *not otherwise explained*, one must pursue a plasma defect or defects as possible causes of the disease. There are many tests of varying complexity which can help delineate these defects. Some of these have been elaborated in the section on technic and others will be mentioned here briefly in the following outline:

1. The Quick prothrombin consumption time or the serum prothrombin time. Hemophiliacs show a very short (± 10 seconds) time. Hemophilia-like states may show a *shortened* or almost *normal time*. This test is nonspecific insofar as the plasma factors are concerned.

2. Standard prothrombin activity tests, e.g., Quick one-stage or modifications thereof, show normal times in classical hemophilia but may be abnormal in some of the hemophilia-like diseases.

3. Owren technics for specific factors, such as prothrombin, proconvertin, and proaccelerin, demonstrate specific defects in hemophilia or hemophilia-like disease. It was by the use of such technics that Owren discovered parahemophilia (or factor V) deficiency.

¹Dameshek, W.: *Blood* 11: 244, 1954.

²Aggeler, P. M., White, S. G., and Spaet, J. H.: *Blood* 11: 246, 1954.

³Feissly, R.: *Blood* 11: 253, 1954.

⁴Brinkhous, K. M., and Graham, J. B.: *Blood* 11: 254, 1954.

⁵Macfarlane, R. G.: *Blood* 11: 256, 1954.

⁶Quick, A. J.: *Blood* 11: 265, 1954.

⁷Stefanini, M.: *Blood* 11: 273, 1954.

⁸Tocantins, L. M.: *Blood* 11: 281, 1954.

⁹Koller, F.: *Blood* 11: 286, 1954.

¹⁰Pavlovsky, A.: *Blood* 11: 291, 1954.

4. Thromboplastin generation test¹ is helpful in distinguishing and assaying various components of the thromboplastin-producing system.

5. Antihemophilic globulin (AHG) assay.² This test measures the specific defect in hemophilia and is useful in detecting latent subclinical cases. Normal AHG assays at 100 per cent, whereas classical hemophilia assays at 0 per cent, moderate hemophilia less than 3 per cent, mild hemophilia 16 per cent, and subhemophilia 33 per cent.

6. The partial thromboplastin test (PTT). This is a screening procedure in which the plasma of patients with hemophilia and hemophilia-like disease may be brought under scrutiny. The authors² have hypothesized the following diagnostic groups: hemophilia, hemophilioid state A, hemophilioid state B, hemophilioid state C, and hemophilioid state D. In all of these the routine PTT gives a prolonged time. However, if equal parts of fresh normal barium-sulphate-treated plasma are added to the test plasma, the results aid in differentiation of the states. With such "fortified" plasmas, in hemophilia and hemophilioid state A the partial thromboplastin time returns to normal but in hemophilioid states B, C, or D, the partial thromboplastin time remains prolonged.

These authors have further elaborated their detection and separation system by making standard prothrombin time determinations on untreated plasma and fortified plasma (see Table 72).

TABLE 72

PROTHROMBIN TIME		
	UNTREATED PLASMA	"FORTIFIED" PLASMA
Hemophilia	Normal	Normal
Hemophilioid state A	Prolonged	Normal
Hemophilioid state B	Prolonged	Prolonged
Hemophilioid state C or D	Normal	Normal

7. Matching experiments. The basic principle of such tests is determining whether mixing the blood of one individual and that of another corrects a known defect. Such matching experiments originally aided in delineating true hemophilia from some of the subvarieties. It was found that in these subgroups, the patients' blood would correct the defect in hemophilia in the same manner as normal blood. Since then, this procedure has become standard practice in the study of hemophilia and related disorders. This technic is limited to separating the entities but does not delineate the specific deficiency. However, an understanding of such matching experiments is helpful in determining therapy. As an example, classical hemophilia will respond to concentrated antihemophilic globulin administration but other coagulation factor deficiency diseases will not respond favorably.

A typical series of such matching experiments may be found in the paper by Brinkhous and associates.³

One of the difficulties in matching experiments has been the need for blood from a patient with the deficiency of plasma factor. As the various

¹Biggs, R., and Douglas, A. S.: *J. Clin. Path.* 6: 23, 1953.
²Brinkhous, K. M., Langdell, R. D., Penick, G. D., and Graham, J. B.: *J. A. M. A.* 154: 481, 1954.
³Brinkhous and others: *J. A. M. A.* 154: 481, 1954.

factors become elaborated, their reproduction in the test tube is becoming more frequent, making such experiments available to more observers. Ideally, the isolation and purification of factors by physical and chemical means would help considerably. Such isolation is understandably difficult when one considers the complexity of the coagulation mechanism.

TABLE 73.—SUMMARY OF CLINICAL AND CLOTTING CHARACTERISTICS OF AHG, PTC, AND PTA DEFICIENCIES*

	ANTIHEMOPHILIC GLOBULIN (AHG) DEFICIENCY	PLASMA THROMBO- PLASTIN COMPONENT (PTC) DEFICIENCY	PLASMA THROMBO- PLASTIN ANTECEDENT (PTA) DEFICIENCY
Sex	Male (female very rarely)	Male	Male and female
Hereditary pattern	Sex-linked, recessive trait carried by female, transmitted to male	Similar to AHG deficiency	Not sex-linked, transmitted to male and female by female and probably male carriers
Pattern of hemorrhage: Degree Sites	Depends upon clotting time and the degree of the clotting defect		
	Slight to very severe Joints frequent, follows trauma and any operative procedure	Slight to moderately severe Joints sometimes involved, follows trauma and operative procedures	Slight to moderate Joints rarely involved, most usually follows minor surgical procedures as tonsillectomy, tooth extraction
Clotting time	Normal to over 1 hour	Normal to about 60 minutes	Normal to 30 minutes
Clotting defect corrected by:			
Normal plasma	Yes	Yes	Yes
Normal BaSO ₄ -treated plasma	Yes	No	Yes
Normal serum	No	Yes	Yes
Fraction I	Yes	No	No
Normal BaSO ₄ -treated serum	No	No	Yes
Other findings	Normal values—platelet count, prothrombin time, clot retraction Usually normal—bleeding time, tourniquet test Abnormal (degree related to the extent of the deficiency)—heparin clotting time, serum prothrombin time, recalcified clotting time of slowly and rapidly centrifuged plasma, thromboplastin generation		

*From Rosenthal, R. L.: *Am. J. Med.* 17: 57, 1954. By permission of author and publisher.

As a result of studies such as those mentioned have come such names as Christmas disease,¹ parahemophilia,² hemophilioid states A, B, C, D,³ PTC,⁴ plasma thromboplastin antecedent (PTA) deficiency.⁵ In addition, there have been designations of various factor deficiencies by number, such as factor V, VI, or VII deficiencies. There is no unanimity of opinion as to which system of nomenclature to adopt and often there is some question as to whether or not authors using different terminology are discussing the same syndrome.

Rosenthal⁶ presents an enlightening article on the clinical, laboratory, and hereditary patterns of this group of diseases. Through his permission and

¹Biggs, R., Douglas, A. S., MacFarlane, R. G., Dacie, J. V., Pitney, W. R., Merskey, C., and O'Brien, J. R.: *Brit. M. J.* 2: 1378, 1952.

²Owren, P. A.: *Lancet* 1: 446, 1947.

³Brinkhaus and others: *J. A. M. A.* 154: 481, 1954.

⁴Aggeler, P. M., White, S. G., Glendening, M. B., Page, E. W., Leake, J. B., and Bates, G.: *Proc. Soc. Exper. Biol. & Med.* 79: 692, 1952.

⁵Rosenthal, R. L., Dreskin, O. H., and Rosenthal, N.: *Proc. Soc. Exper. Biol. & Med.* 82: 171, 1953.

⁶Rosenthal, R. L.: *Am. J. Med.* 17: 57, 1954.

that of his publisher, we reproduce a table from this communication which presents an excellent bird's-eye view of plasma factor deficiencies, Table 73.

Whatever else may be said about these new entities, there is no question that this elaboration has added considerably to knowledge related to the coagulation mechanism.

BONE MARROW STUDIES

Many of the hematologic observations which Schilling and other modern hematologists have made, and are now making, are based upon bone marrow studies. In all uncertain cases of blood diagnosis, it is well to obtain some of the bone marrow for diagnostic examination.

In the human adult the bone marrow of the long bones is yellow; the marrow is pink **at birth**. In the early years, say about **seven years**, fat in small quantities appears; between **twelve** and **fourteen years**, a visible patch of yellow appears in the middle of the femur which increases until there is only a small patch of pink marrow near the end of the diaphysis in the **adult**. The same phenomenon takes place in the tibia, ulna, radius, humerus, and fibula. The only red marrow which persists in the adult is to be found in the bones of the skull, ribs, vertebra, sternum, and the innominate bones. Bone marrow studies, therefore, are most conveniently made from the adult sternum.

The bone marrow in infants shows many immature cells. In the **six months' fetus** the red cells make up three-quarters of the total; **at eight months**, nine-tenths; and **at term**, the red and white cells are equal. Latimer and Mayerhoff found fetal marrow containing 2 to 22 per cent granulocytes. Doan and Zerfas state that during the first year of life the marrow contains 4 to 20 per cent lymphocytes. In embryonic life the ratio of erythroid to myeloid elements is 100 to 0. In adults, it is 1 to 3. Since the red bone marrow of newborn children contains many more immature cells than adult bone marrow, it is easy to understand the ready appearance of immature cells in the circulating blood of an infant following slight stimuli. In children, the number of cells in the bone marrow varies from 270,000 to 1,568,000 per c.c. In adults we find in the total bone marrow volume of 1,500 c.c. in the human body that the myeloid elements outnumber the erythroid elements 3 to 1; most of the myeloid cells, 85 per cent, are the myelocytic cells with their full quota of granules. There is a diurnal variation in leukocytes with an afternoon and midnight rise. The bone marrow delivers granulocytes, myelocytes, juveniles, and "stabs" hourly, but especially at the interval at which the leukocytes in the blood stream are increased. The maturation time from myelocytes to more mature forms is probably from forty-five minutes to two and one-half hours. One per cent of the bone marrow elements are megakaryocytes. According to Wright, these constitute the origin of blood platelets, but according to Schilling's ideas, blood platelets do not come from megakaryocytes but are extruded remnants of nuclei of red blood cells. In bone marrow, 70 per cent of the total erythroid cells are normoblasts; erythroblasts, 26 to 30 per cent; proerythroblasts, 1 to 4; and megaloblasts, 0.01 to 0.04. According to Schilling, the megaloblasts represent a stage in the development of the red cells, but according to Ferrata and Michaelson,

the megaloblast is present in the bone marrow only in cases of pernicious anemia. They do state, however, that the cell which is sometimes mistaken for the megaloblast is in reality the proerythroblast. Sabin and Doan corroborate Schilling's claim that the megaloblast is present in normal bone marrow, but only in very small numbers.

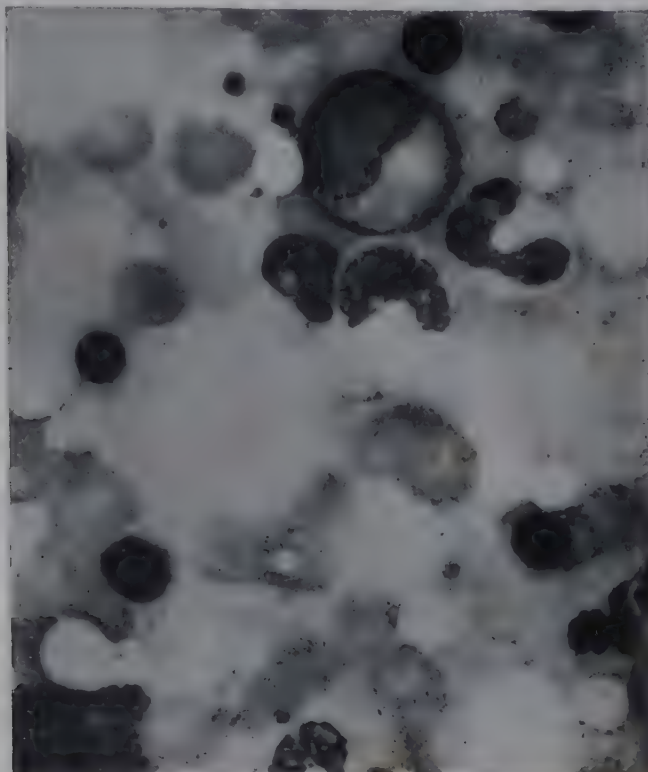


Fig. 216.—Normal bone marrow. Note lack of crowding of cells and the presence of fat. (×950.)

Reticulated red cells and normoblasts are released into the blood stream at regular intervals; the red cell count varies about 1,000,000 per cu. mm. per day. The high point of bone marrow delivery is in the morning and afternoon, not afternoon and midnight, as is the case with the white blood cells.

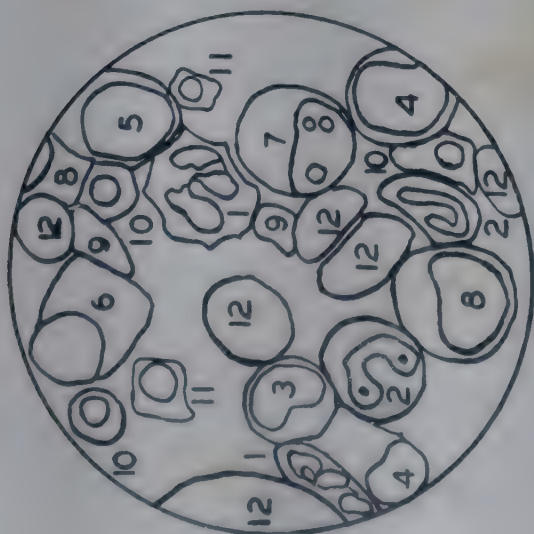
Summary of Normal Bone Marrow Findings

What is the normal cellular content of the bone marrow? We must assume, first, that under normal conditions, myelopoiesis and erythropoiesis are confined to the bone marrow. Lymphocytopoiesis normally does not seem to be confined to the bone marrow. The investigations of Askanazy, Oehme, von Fischer, and others showed that there are fully developed lymph follicles in the bone marrow in a large number of cases. Under normal conditions, however, lymphocytopoiesis seems to have no connection with either erythropoiesis or myelopoiesis although there is no objective hematologic proof of this. The Schilling followers do not believe that monocytopenia is connected with bone marrow function. Monocytes are seen in very few numbers in bone marrow according to Schilling, Sabin, and others. Yamamoto and Schilling¹ have shown in man and in animals that the composition of different parts of the marrow is practically the same on the same occasion in the same individual. In a few cases a peripheral eosinophilia occurred which was accompanied by an increase in the proeosinophiles in the bone marrow. On the other hand abnormally low

¹Yamamoto and Schilling: *Virchows Arch.* 258: 62, 1925.

BONE MARROW TOUCH PREPARATIONS

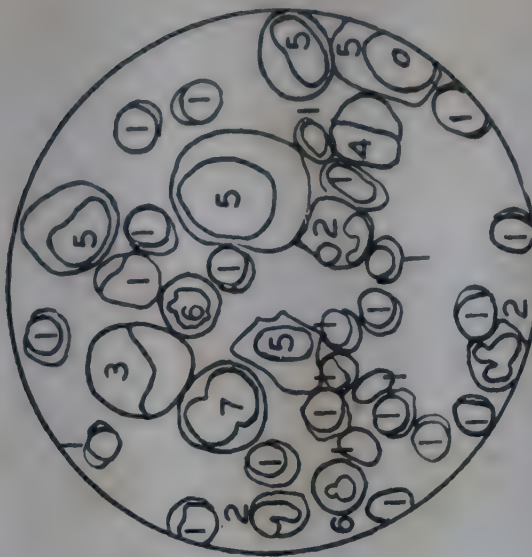
WRIGHT-GIEMSA STAIN X950



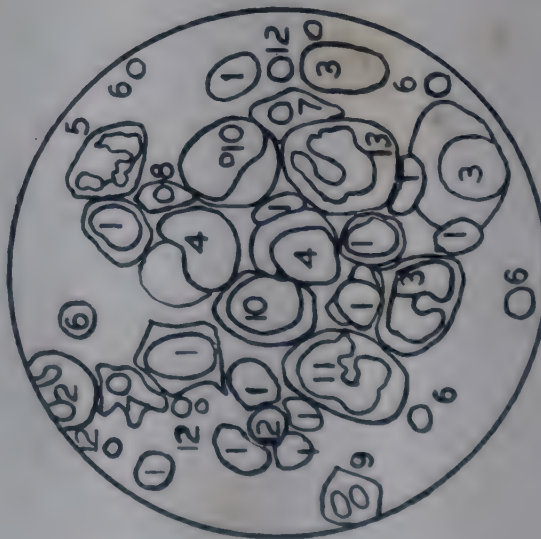
NORMAL



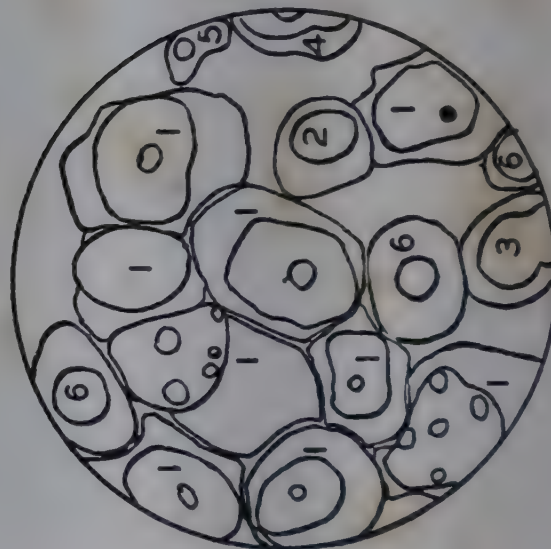
PERNICIOUS ANEMIA



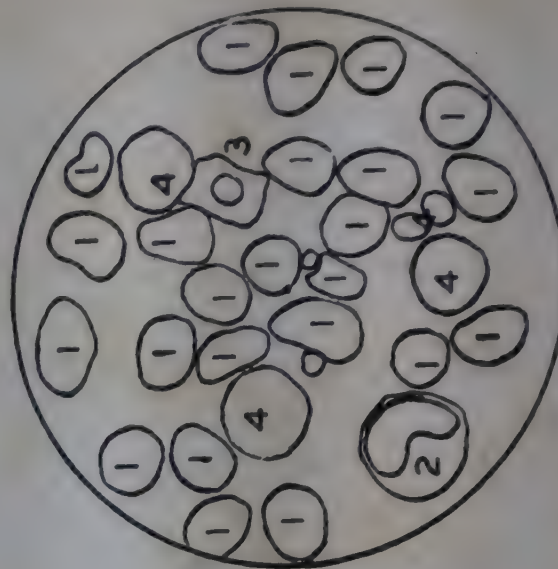
APLASTIC ANEMIA



CARCINOMA OF THE LIVER



MYELOCYTIC LEUKEMIA



LYMPHOCYTIC LEUKEMIA



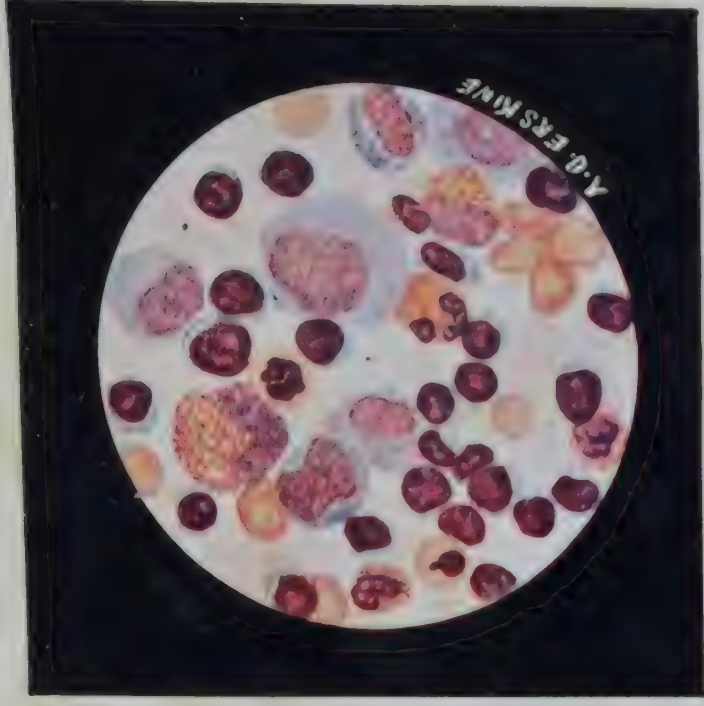
NORMAL



CARCINOMA OF THE LIVER



PERNICIOUS ANEMIA



APLASTIC ANEMIA



LYMPHOCYTIC LEUKEMIA



MYELOCYTIC LEUKEMIA
PLATE XXV.

PLATE XXV.—BONE MARROW TOUCH PREPARATIONS

*Wright-Giemsa Stain ×950**Normal*

1. Segmented neutrophile
2. "Stab"
3. Neutrophilic juvenile
4. Neutrophilic myelocyte
5. Basophilic myelocyte
6. Eosinophilic myelocyte
7. Promyelocyte
8. Myeloblast
9. Polychromatic erythrocyte
10. Orthochromatic normoblast
11. Polychromatic normoblast
12. Fat

Carcinoma of the Liver

1. Small lymphocyte
2. Eosinophile
3. Young cells, probably myeloblasts
4. Promyelocyte
5. Segmented neutrophile
6. Nuclei—probably of normoblasts
7. Orthochromatic normoblast
8. Polychromatic normoblast
9. Polychromatic normoblast with karyorrhexis
10. Myeloblasts
11. Basophile
12. Fat
13. Neutrophilic juveniles

Pernicious Anemia

1. Polychromatic megaloblast
2. Polychromatic macroblast
3. Orthochromatic macroblast
4. Segmented neutrophile
5. Eosinophilic myelocyte
6. Neutrophilic myelocyte
7. "Stab"
8. Atypical cell—young giant neutrophilic cell

Aplastic Anemia

1. Small lymphocyte
2. Eosinophile
3. Eosinophilic juvenile
4. Eosinophilic myelocyte
5. Myeloblast or lymphoblast (♀)
6. Orthochromatic normoblast with karyorrhexis
7. Young lymphocyte

Lymphocytic Leukemia

1. Lymphocyte
2. Neutrophilic juvenile
3. Orthochromatic normoblast
4. Fat

Myelocytic Leukemia

1. Myeloblast
2. Eosinophilic myelocyte
3. Neutrophilic myelocyte
4. "Stab"
5. Orthochromatic normoblast
6. Orthochromatic macroblast

TABLE 74.—BONE MARROW CONTENT OF NORMAL INDIVIDUALS ACCORDING TO VARIOUS AUTHORS

	DOAN AND ZERFAS	ZADEK	YAMA- MOTO	SCHILLING AND BENTZLER	VARELA	BARTA	WEINER AND KAZ- NELSON	ARINKIN	TEMPKA AND BRAUN	NORDENSON (THE PRESENT MATERIAL)
Myeloblasts	1.1-11.9	8	2	-	5.54	2-3	4.6	1.0- 2.4	4.66- 7.0	0.25- 5.50
Metamyeloblasts	-	-	-	-	-	-	-	-	1.50- 5.50	-
Promyelocytes	-	14	7	-	8.4	6-8	5	1.0- 2.8	3.75- 6.83	1.25- 8.25
Myelocytes	18.8-54.9	25	44.5	34.8-47.4	0.57	-	-	-	0.55- 0.66	-
	0.1- 4.1	18	4.5	-	20.55	40-42	19.4	4.5- 8.6	12.75-13.33	4.25-18.0
	0- 0.1	-	-	-	0.94	-	1	0.3- 1.0	1.50- 2.66	0- 6.25
		-	-	-	0.22	20-22	0.2	-	0- 0.33	0- 0.50
Metamyelocytes	-	22	22.5	34.0-56.2	27.43	-	15.7	1.4- 3.4	14.33-16.25	12.50-42.50
	-	-	-	-	0.73	-	-	0.3- 1.0	0.33- 3.66	-
Rod forms	-	-	-	0-1	-	6-8	63	-	0- 0.16	-
	-	-	9	-	-	-	-	-	17.0-22.50	2.25-10.75
	-	-	-	-	-	-	-	-	0.50- 1.0	-
Segmented forms	-	-	-	-	-	-	-	-	0- 0.16	-
	10.5-55.4	6	8.5	7.0-22.0	-	8-10	22.3	41.5-55.0	16.16-20.33	14.25-35.0
Lymphocytes	0- 0.6	-	1	1.1- 4.6	-	-	2.1	0.6- 4.0	0.66- 2.50	0.25- 7.50
Monocytes	0- 0.1	-	-	-	-	-	0.8	0- 0.7	0.16- 0.33	0- 0.75
Plasma cells	10.8-37.3	7	-	-	-	-	13.6	7.3-16.5	2.66- 3.25	7.5-38.0
Clasmatocytes	0.5- 4.5	-	-	-	-	-	6.5	2.1- 9.3	0.50- 0.75	0-5
Unclassified	0.1- 0.27	-	-	-	-	-	2.1	0.3- 0.9	0.25- 1.66	0- 3.25
Megakaryocytes and megakaryoblasts	0.3- 1.1	-	-	11.8-24.8	-	-	-	-	-	-
Primitive cells	-	Numerous	-	-	-	-	-	-	0-3	0- 4.50
Ferrata cells	0- 0.45	-	-	-	0.29	-	0.2	0.6- 6.1	2.16-4	0-1
Endothelial cells	0.4- 1.1	-	-	-	-	-	-	-	-	-
Histiocytes	-	-	-	-	-	-	0.2	-	1.33- 3.75	3-40
Hemocyotoblasts	-	-	-	-	-	-	-	-	-	-
Megaloblasts	-	-	-	-	0.86	-	-	-	0.50- 1.0	-
Pronormoblasts	0- 0.1	-	-	-	2.23	-	-	-	-	-
Basophilic normoblasts	-	-	-	-	-	-	-	-	-	-
Normoblasts	-	-	-	-	-	-	-	0.8-2.9	-	0-6
	4.1-13	Numerous	-	-	5.53	-	-	-	-	1-16
			-	31.6-43.7	21.71	-	25.6	5.7-16.0	-	26-184

*From Nordensen, *Studies on Bone Marrow from Sternal Puncture*, Generalstabens Litografiska Anstalts Förlag, Stockholm.

numbers of peripheral eosinophiles were not represented by low values in the marrow. According to these authors parallelism does not exist between peripheral and bone marrow eosinophilia. Homma² found a constant bone marrow eosinophilia with an elevation in the number of peripheral eosinophiles. But conversely there may be normal or somewhat increased numbers of eosinophiles in the bone marrow in spite of peripheral decreased number of eosinophiles. Magne, in his work entitled "La Grande Eosinophilie Sanguine" (Thèse, Lyon, 1933), substantially is in accord with Homma. Barta found that the effect of toxin on eosinophiles is exerted on the fully developed cells which have wandered out and not on the young forms in the marrow which explains the peripheral decreased number of eosinophiles without a corresponding decrease in the eosinophiles of the marrow. Table 74 from "Studies on Bone Marrow From Sternal Puncture" by Nils Göran Norden-son, published by Generalstabens Litografiska Anstalts Förlag, Stockholm, gives the composition of bone marrow according to a number of authorities.

Holmes and Broun³ call attention to the mitotic divisions of cells in the bone marrow spreads made by them as being limited to an intensely basophilic cell which varies in morphology from both the typical myeloblast and the typical megaloblast. They have called this cell the primitive cell in their differential counts. They occasionally noted mitotic division in hemoglobin-containing erythroblasts. **Differential counts** by these writers of **bone marrow elements** in seven cases with normal peripheral blood pictures were as follows: myeloblasts 2.4 per cent; myelocytes 7 per cent; juveniles, 6.7 per cent; "stabs" 14 per cent; II segmented neutrophils 10 per cent; III segmented neutrophils 6.3 per cent; IV, V, etc., segmented neutrophils 1.1 per cent; eosinophiles 1 per cent; basophiles 0.3 per cent; monocytes and reticuloendothelial elements 9 per cent; lymphoid cells 24.9 per cent; megaloblasts 5.2 per cent; normoblasts 6.9 per cent; primitive cells 2.6 per cent. These findings agree rather closely with those of Arinkin⁴ on similar types of cases.

It is interesting to note that the high percentage of immature granulocytes in their counts confirms the concept of Arneth and Schilling that the neutrophilic nucleus changes progressively with age from the round to the segmented form. The high percentage of juveniles and stabs confirms the idea that these are "young" granulocytes.

An examination of Table 73 and similar tables in other publications reveals wide variations in opinions on the relative percentages of various cells, and raises some question as to the value of doing differential counts routinely on bone marrows. We are inclined to agree with Israels⁵ that "the variability of the normal bone marrow means, in effect, that only gross differences can be taken to indicate pathological change, and this in turn raises a question of whether it is worth while to undertake, in every case, the time-consuming differential count . . . this practice has now been given up for a 'qualitative assessment' in all except the most difficult cases, or in research studies. Such

²Homma: Virchows Arch. 233: 11, 1921.

³Holmes, W. F., and Broun, G. O.: Proc. Soc. Exper. Biol. & Med. 30: 1306-1308, 1933.

⁴Arinkin, M. J.: Klin. Med. 7: 135, 1929.

⁵Israels, M. C. G.: An Atlas of Bone Marrow Pathology, New York, 1948, Grune & Stratton, p. 27.

a qualitative assessment is adequate for diagnosis in most intance, and gives a far clearer idea of the overall marrow picture than does a row of figures, especially since few physicians or pathologists carry the normal figures around with them.” We have used an assessment type of reporting clinical marrow aspirations, and include the following data in such reports:

Cellularity—“normal,” “increased,” or “decreased.”

Predominant cell type—normally this should be members of the myelocytic series.

Myelocytic series—normally shows predominance of older forms, e.g., stabs and segments, with a gradual diminution to myeloblasts. If a left shift is present, comment is made on the degree. Qualitative changes in the cells of this series are described, if present. Such changes include toxic granules and abnormal forms. The myeloid-erythroid ratio is estimated.

Erythrocytic series—normally shows predominance of older forms, e.g., orthochromic normoblasts with a scattering of younger forms. If a shift has occurred to younger members of this series, its degree is appraised and reported. The type of erythropoiesis is described, e.g., “definitive” (as in normal red blood cell development) or “primitive” type (as in pernicious anemia and some other markedly pathologic states). Qualitative changes, indicating pathologic erythropoiesis, are noted. These include marked poikilocytosis (poikilocytes, spherocytes, target cells), anisocytosis, basophilic stippling, abnormalities of nuclear morphology (karyorrhesis, karyolysis), and abnormal relationships of nucleus to cell size.

Lymphocytic series—normally less than 10%. Quantitative or qualitative changes in these cells are noted.

Megakaryocytes—normally readily found, especially in the thin and thick ends of spreads. They tend to be fewer in the middle of spreads. Qualitative changes, such as cytolysis with degree of platelet formation, are described.

Other cells, such as fat, reticuloendothelial cells, and “cells foreign to normal bone marrow (tumor cells)” are noted.

Mitoses are noted as to frequency, and comment is made as to whether mitotic figures are normal or abnormal in appearance.

TABLE 75.—BONE MARROW: GENERAL COMPARISON OF SPREADS AND SECTIONS

	SPREADS	SECTIONS
Cellularity	Varies with technic	Consistent with true state of marrow
Relative distribution of cell species	Irregularities dependent on technic	Accurate reflection
Identification of individual cells	Better than in sections	More difficult than in spreads
Mitotic activity	Morphology of individual mitotic figures better than in sections	Quantitative aspect more readily apparent than in spreads
Histologic structure	Only implied	Readily discerned
Cytochemical studies	Material unsatisfactory	Material satisfactory
Lymphomata	Easily missed or not apparent	Readily identified
Granuloma lesions	Not seen	Readily identified
Maligancy	Individual cells or groups of cells irregularly distributed	Syncytial masses readily found; histologic arrangement may indicate source

Such appraisals are not based on spreads alone. Agress¹ has shown the value of studying sections (see page 943 for technic) and spreads simultaneously in order to obtain a true quantitative evaluation of bone marrow. A general comparison of the two methods is shown in Table 75 by Agress, which is based on his study of 1,187 cases in which bone marrow aspirations were performed.

¹Agress, H.: Comparative Study of Bone Marrow Spreads and Bone Marrow Sections, Exhibit at Clinic Session, A.M.A., Miami, Fla., 1954.

As can be readily noted from Table 75, many differences are apparent between spreads and sections. Perhaps the outstanding difference relates to quantitative appraisal.

It is not unusual to find spreads which appear extremely hypocellular, whereas the sections may appear quite normally cellular. On the other hand, individual cell morphology is more clearly studied in well-prepared spreads.

In this study, a correlation between spreads and sections of bone marrow was set up by Agress in relation to their being diagnostic. Table 76 represents this correlation.

There is close correlation between spreads and sections insofar as the over-all percentage in satisfactory preparations is concerned. However, in individual cases, the spread may be unsatisfactory and the section satisfactory. In other instances, the reverse may be true. This correlation is particularly important in the hypoplastic or aplastic states. Note that no cases of aplastic anemia were diagnosed on spreads alone and only 20 per cent were

TABLE 76.—BONE MARROW: CORRELATION OF PREPARATIONS

	SPREADS	SECTIONS
	%	%
Per cent satisfactory preparations	92	95
Anemia, etiology variable	0*	8*
Aplastic anemia	0*	20*
Carcinoma	90*	100*
Granuloma lesions	0*	100*
Histiocytes	100	0
Hodgkin's disease	3*	8.7*
Leukemia	100*	100*
Leukosarcoma	60*	90*
Lymphosarcoma	9.4*	100*
Multiple myeloma	72*	97*
Myelofibrosis	0*	33*
Pernicious anemia	95*	94*

*% Diagnostic.

diagnosed on sections of the clot obtained by routine marrow aspirations. The reason for this should be obvious. In those cases of aplastic anemia in which the bone marrow is almost completely replaced by fibrous tissue, routine marrow aspirations are generally doomed to failure. This diagnosis should not be made on the **absence** of cells from the spread **alone**. If sections of clot show no marrow units, the diagnosis still should not be made since the result may be purely technical in origin. In such an event, the marrow aspiration must be repeated or surgical trephine should be performed to get a truly representative specimen of bone marrow. This figure also gives some suggestion of what the physician might expect diagnostically from bone marrow aspiration.

Like all other laboratory examinations, marrow studies have their limitations and serve their most useful purpose only when correlated with the clinical aspects of the patient. Bone marrow aspiration should not be performed prior to a thorough history, physical examination, and at least routine laboratory studies. This is particularly true in the vast majority of anemias, except pernicious anemia and a few others. In relation to pernicious anemia and related macrocytic anemias, it is important to remember that erythrocyte maturation factor in even modest dosages will convert the bone marrow pic-

ture considerably. For this reason, folic acid, liver, and vitamin B₁₂ should not be administered prior to marrow aspiration. Table 77 by Agress is an outline of the indications for marrow aspirations. The cases listed under "hypersplenism"¹ are particularly to be stressed since this is the group in which splenectomy may exert a very favorable influence.¹ It is well to remember that selected cases displaying the features of hypersplenism may be primarily due to metastatic carcinoma of the bone marrow. Splenectomy could serve no useful purpose in such cases. For this reason, bone marrow aspiration should be performed in all cases in which splenectomy is contemplated as a form of treatment for a blood dyscrasia. It has been our experience generally that the best results in this group of cases come in those patients whose bone marrow contains a normally active or hyperactive picture of the blood element or elements affected. For example, we do not recommend splenectomy in a case of idiopathic thrombocytopenic purpura unless the bone marrow shows a fairly good representation of megakaryocytes. Using this criterion alone our

TABLE 77.—INDICATIONS FOR MARROW ASPIRATION

-
- | |
|--|
| 1. Every case of hypersplenism |
| a. Thrombopenia |
| b. Hemolytic anemia |
| c. Leukopenia |
| d. <i>Emphatically</i> when (a), (b) and (c) are combined. |
| (Splenectomy should not be performed in these cases without examination of bone marrow.) |
| 2. All cases with a persistent bizarre blood picture. |
| 3. Cases in which there is a high index of suspicion of marrow invasive disease, e.g., carcinoma, myeloma, Gaucher's disease, etc. |
| 4. Corroboration of a limited number of cases of leukemia in which peripheral blood studies are inconclusive. |
| 5. Macrocytic anemias of all types prior to therapy with erythrocyte maturation factor. |
| 6. Malignant lymphomata in which lymph nodes are either unavailable or inconclusive. |
| 7. A limited number of infections, e.g., brucellosis, histoplasmosis, tuberculosis, malaria (very rarely), leishmaniasis. |
| 8. All primary refractory anemias. (Repeated "dry taps" may require trephine.) |
-

results with splenectomy have been exceedingly favorable. Much has been written about the bone marrow picture in idiopathic thrombocytopenic purpura as related to splenectomy, and there is no evidence other than the presence of adequate numbers of megakaryocytes (mature or immature) upon which the many observers will agree.

The second group of cases with a bizarre blood picture forms a rare heterogeneous group that covers the horizon of medicine. It includes individual cases of drug intoxications, familial cell defects, or the unusual reflection of some well-known disease. It may include leukopenias or hyperleukocytosis as well as unusual cells in the peripheral blood. Abnormal forms of erythrocytes as well as unexplained anemias should be included here. Thrombocytopenias or morphologically abnormal platelets ("giant platelets") are included in this group. Many of these cases are associated with organ enlargements, such as the spleen and liver, suggesting some blood dyscrasias. Some

¹Dameshek, W.: *Hypersplenism and Surgery of the Spleen, The Spleen and Hypersplenism*, New York, 1953, Grune & Stratton.

of these cases are examples of "hypersplenism," and as our knowledge of immunohematology broadens, they are being more accurately categorized. Some of these cases are in reality malignancies with or without demonstrable bone marrow metastases.

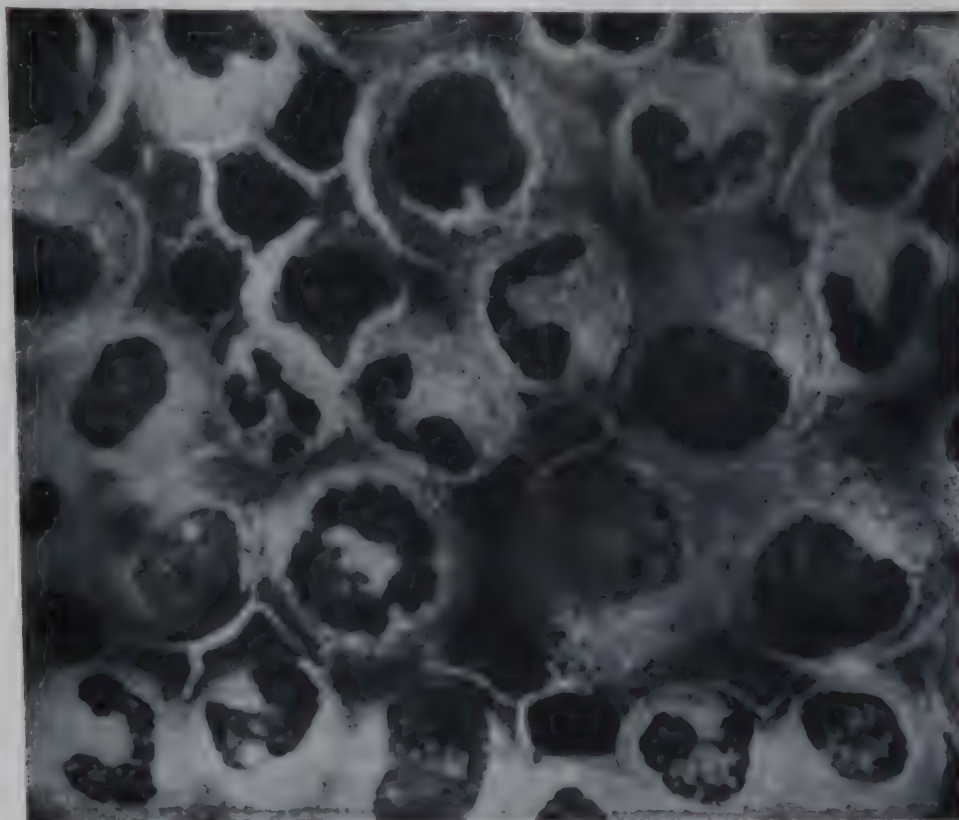


Fig. 217A.—Infection, bone marrow, showing crowding of cells and absence of fat. ($\times 950$.)

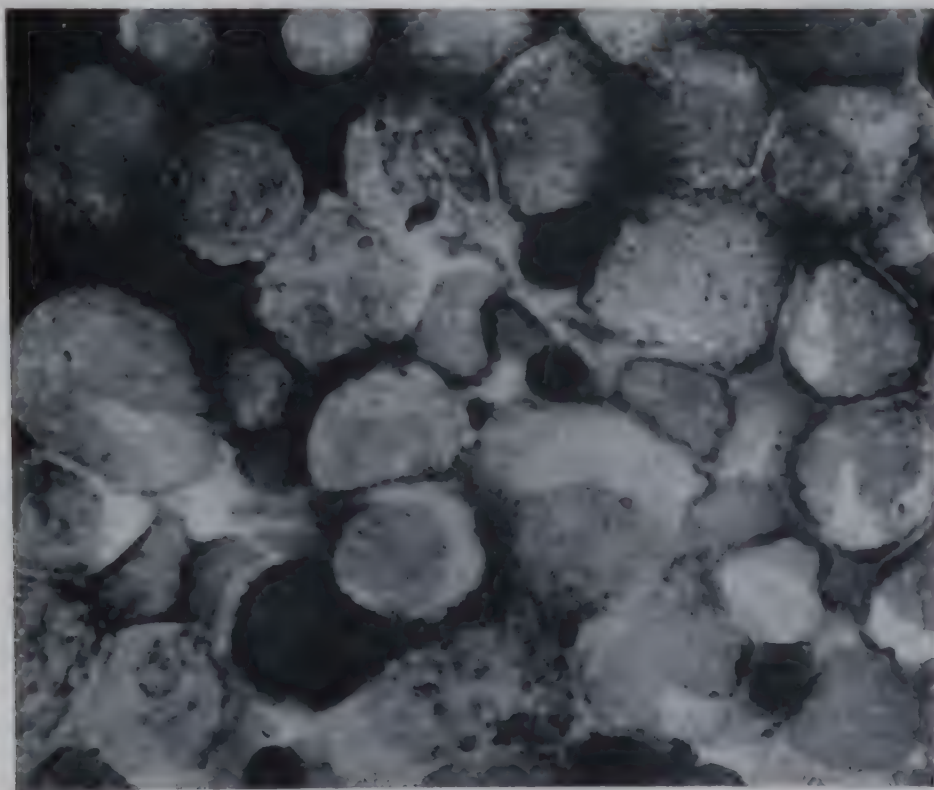
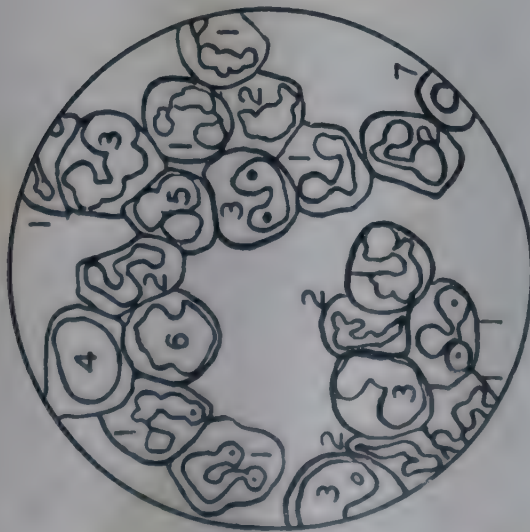


Fig. 217B.—Hyperplastic bone marrow. Note crowding of cells, marked increase of very young white cells, and absence of fat. ($\times 950$.)

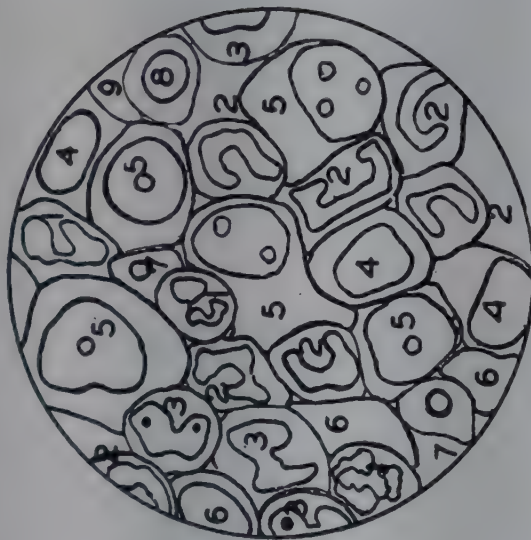
The diagnosis of bone marrow invasion by metastatic carcinoma is dependent upon a number of factors, most important of which is a high index of suspicion. In our experience, the finding of *one or more normoblasts in the*

BONE MARROW TOUCH PREPARATIONS

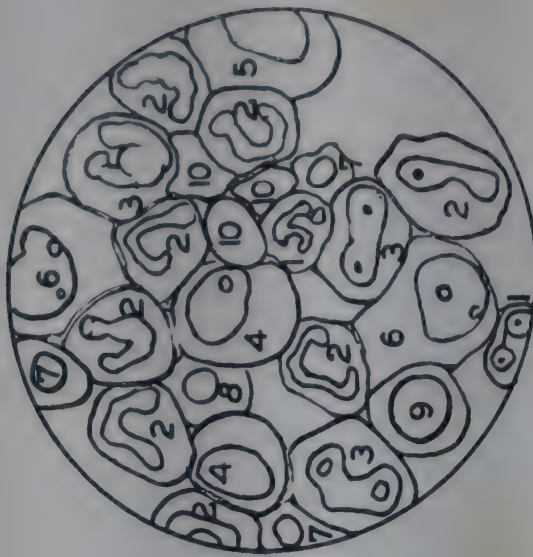
WRIGHT-GIEMSA STAIN X950



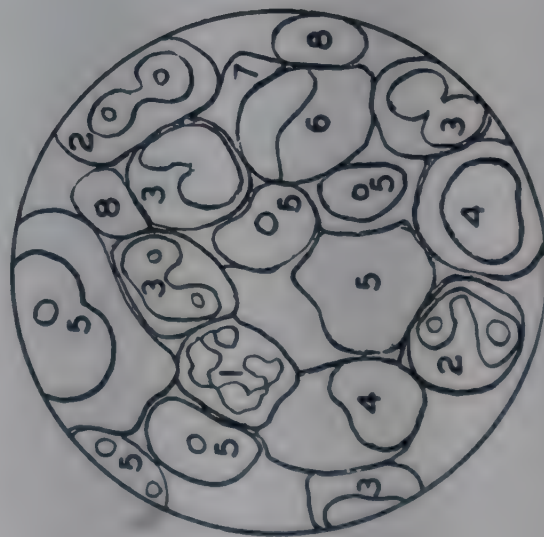
TUBERCULOSIS
BEGINNING "STAB" SHIFT



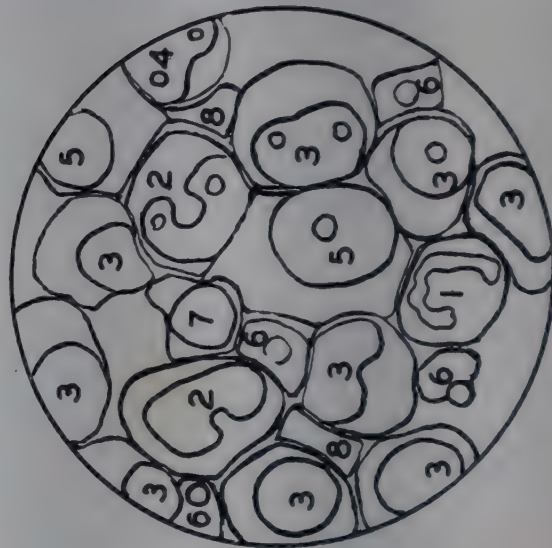
TUBERCULOSIS
"STAB" SHIFT



SEVERE INFECTION

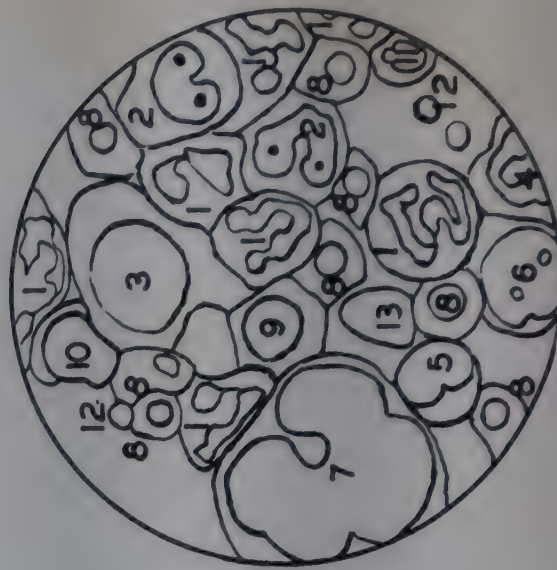


ACUTE INFECTION

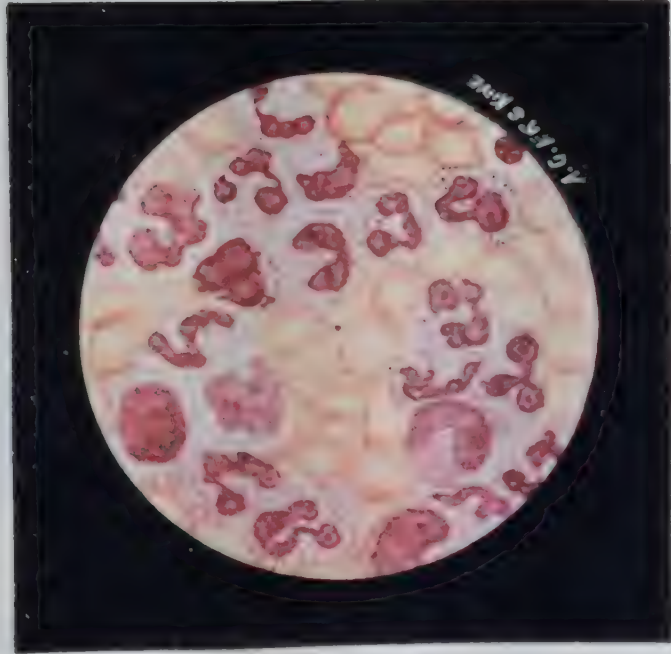


VERY SEVERE INFECTION

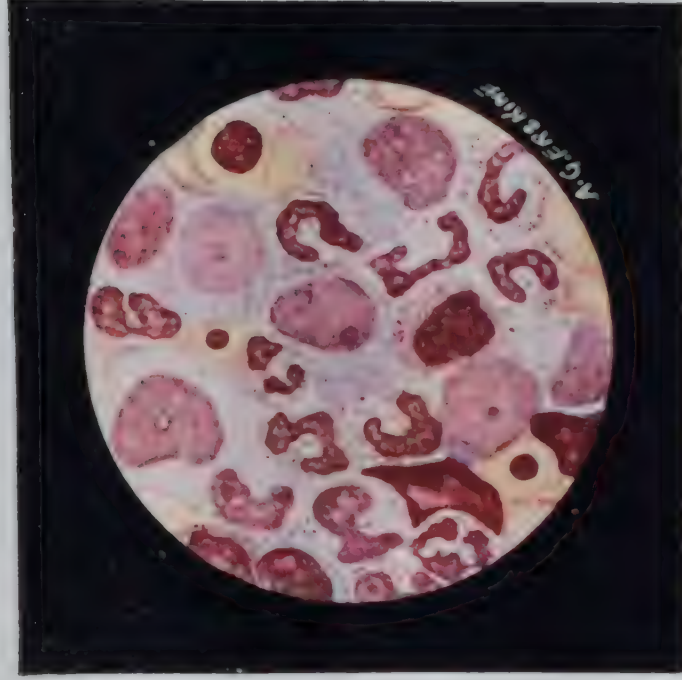
PLATE XXVI



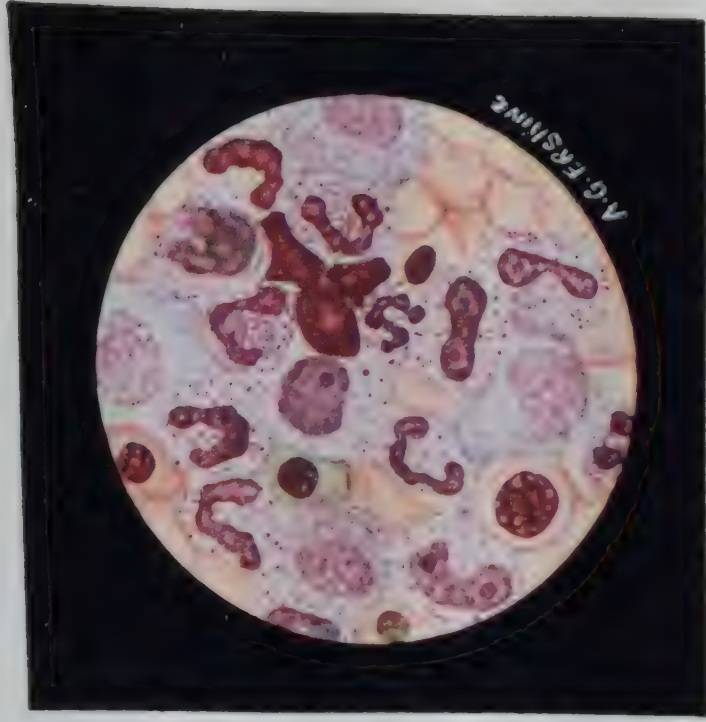
TRYPANOSOMIASIS
GUINEA PIG



TUBERCULOSIS
BEGINNING "STAB" SHIFT



TUBERCULOSIS
"STAB" SHIFT



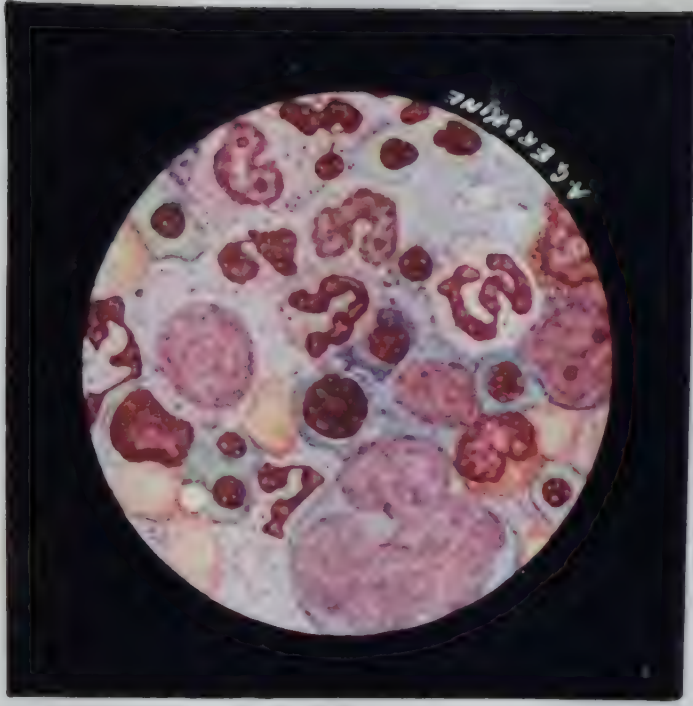
SEVERE INFECTION



ACUTE INFECTION



VERY SEVERE INFECTION



TRYPANOSOMIASIS
GUINEA PIG

PLATE XXVI.—BONE MARROW TOUCH PREPARATIONS

Tuberculosis
Beginning "Stab" Shift

1. Segmented neutrophile
2. "Stab"
3. Neutrophilic juvenile
4. Myeloblast
5. Basophile
6. Monocyte (1)
7. Orthochromatic normoblast

Tuberculosis
"Stab" Shift

1. Segmented neutrophile
2. "Stab"
3. Neutrophilic juvenile
4. Neutrophilic myelocyte
5. Myeloblast
6. Lymphocyte
7. Orthochromatic normoblast
8. Orthochromatic macroblast
9. Polychromatic normocyte

Acute Infection

1. Segmented neutrophile
2. Atypical neutrophilic juveniles about to segment
3. Neutrophilic juvenile
4. Neutrophilic myelocyte
5. Myeloblast
6. Very young eosinophilic myelocyte
7. Lymphocyte
8. Immature nuclear structure

Very Severe Infection

1. "Stab"
2. Neutrophilic juvenile
3. Neutrophilic myelocyte
4. Promyelocyte
5. Myeloblast
6. Orthochromatic normoblast
7. Polychromatic macroblast
8. Lymphocyte

Severe Infection

1. Segmented neutrophile
2. "Stab"
3. Neutrophilic juvenile
4. Neutrophilic myelocyte
5. Promyelocyte
6. Myeloblast
7. Orthochromatic normoblast
8. Polychromatic normoblast
9. Orthochromatic macroblast
10. Lymphocyte

Trypanosomiasis,
Guinea Pig

1. Segmented neutrophile
2. Neutrophilic juvenile
3. Promyelocyte
4. Eosinophile
5. Eosinophilic juvenile
6. Myeloblast
7. Megakaryocyte
8. Polychromatic normoblast
9. Polychromatic macroblast
10. Lymphocyte
11. Polychromatic normoblast with beginning karyorrhexis
12. Fat droplets
13. Nucleus of myeloblast

peripheral blood of an adult not having an obvious disease explaining the presence of normoblasts is one of the most important indicators of possible malignant invasion of the bone marrow. Not all cases of bone marrow malignancy

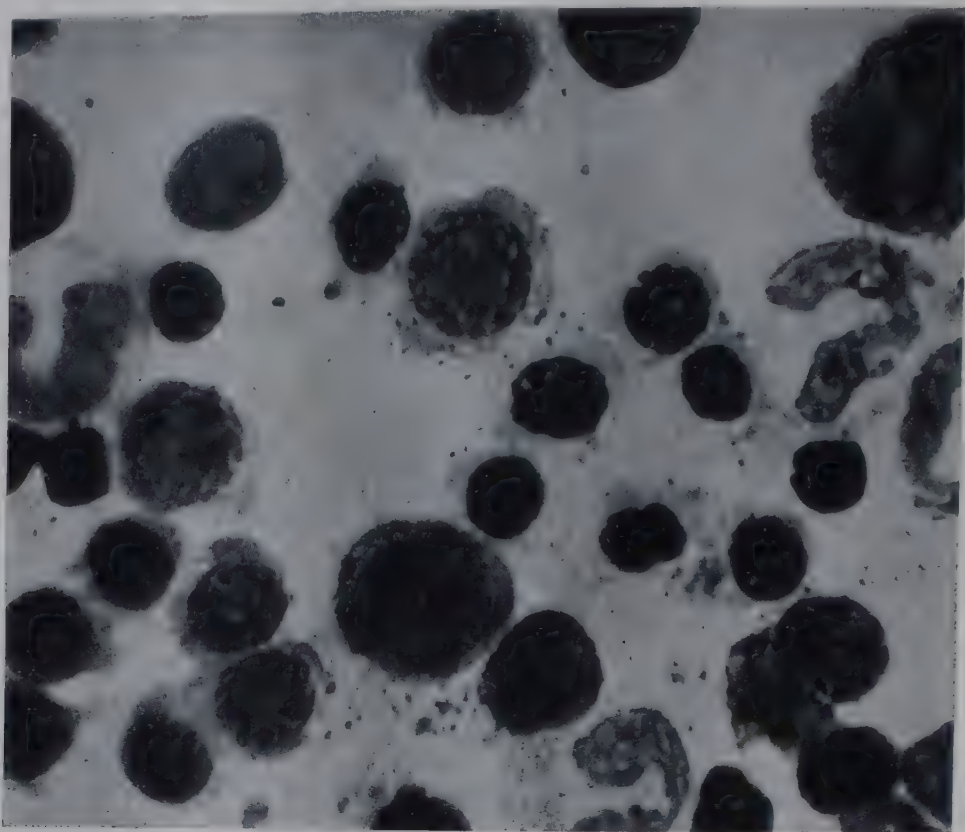


Fig. 218.—Bone marrow, erythropoiesis, showing abnormal number of erythroblasts. ($\times 950$.)

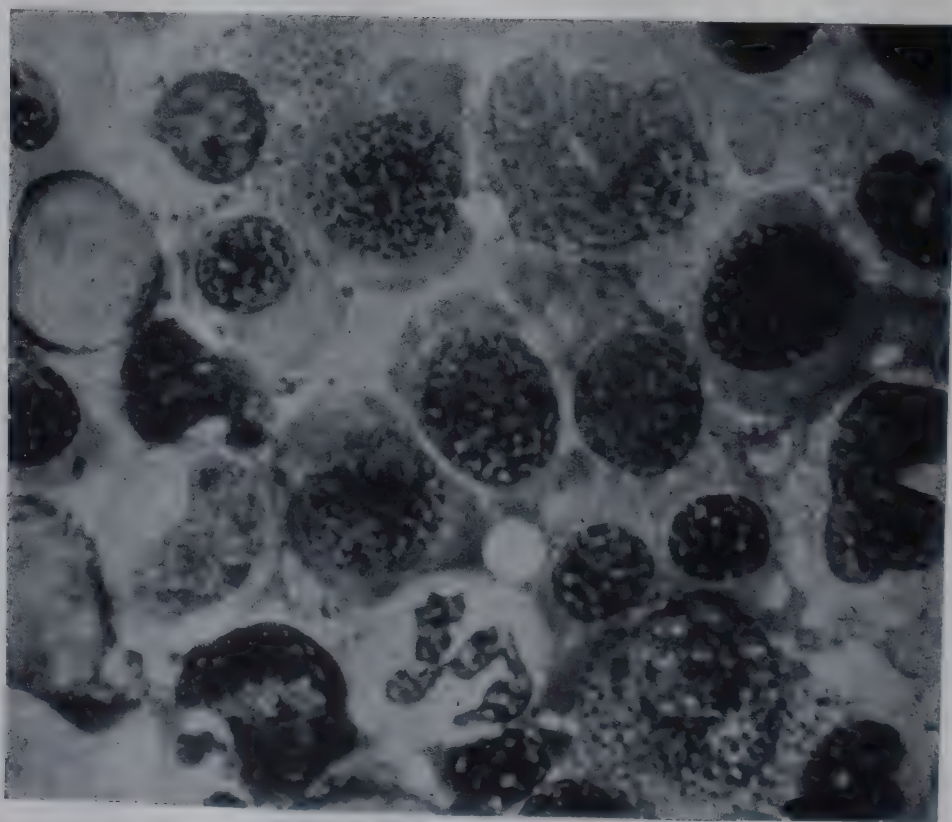


Fig. 219.—Pernicious anemia, bone marrow, showing abnormal number of megaloblasts. ($\times 950$.)

show normoblasts in the peripheral blood, but when normoblasts are present, we pursue the possibility of malignancy vigorously. We have uncovered two cases of unexpected malignancy with normal blood values, in which normoblasts were found on routine blood spreads. The success with which marrow

aspiration will reveal metastases is dependent upon the vigor with which such searches are made. If multiple sites are aspirated and numerous slides examined, the positive results may be very high. Sections of bone marrow clot often will show tumor cells otherwise difficult to find in spreads alone. The section technic outlined by Agress (page 943) may also give some information as to the original site of the malignancy by the histologic structure assumed by the tumor cells.

Bone Marrow Findings in Multiple Myeloma

Multiple myeloma is a form of malignant tumor occurring in the skeletal trunk of adults and infiltrating the marrow cavity of numerous bones. It has been found in people within the age group of 30 to 75 years, the greatest incidence being at the age of 35 years. It affects the ribs and spine and the upper ends of the long bones, but the flat bones of the skull and pelvis may also be implicated. Ewing¹ states that myeloma means a tumor of the specific bone marrow cells, this including lymphocytes, granular leukocytes, and the red cell series, but excluding tumors arising from fatty tissues, blood vessels, and those derived from indifferent endothelial cells. There are four types of myeloma: the plasma cell type, the myelocytoma, the lymphocytoma and the erythrocytoma. Geschickter and Copeland² gave six cardinal signs, two or more of which they thought were constantly present. These were: "(1) Multiple involvement of the skeletal trunk in an adult; (2) pathologic fracture of rib; (3) excretion of Bence-Jones bodies*; (4) characteristic backache with signs of early paraplegia; (5) an otherwise inexplicable anemia; and (6) chronic nephritis with high nitrogen retention, low blood pressure, and high serum proteins." Cases have been reported with none of these signs present. Beizer, Hall, and Giffin³ point to other characteristic signs of this disease, which are: (1) Hyperproteinemia, with reversal of the albumin-globulin ratio; (2) hypercalcemia with normal or high serum phosphorus; (3) evidence of autohemagglutination in the counting chamber, on blood films, or detected by the cayenne pepper appearance of blood in the small vessels of the fundus when viewed through the ophthalmoscope while pressure is being placed on the eyeball; and (4) the appearance of an anticomplementary reaction when a complement-fixation test is being carried out. That none of these signs is pathognomonic is obvious.

Mistakes are easy when dealing with a supposed case of multiple myeloma. These cases may be mistaken for metastatic skeletal lesions, hyperparathyroidism, spondylitis, nephritis, and leukopenic leukemia, or vice versa. Quite frequently the suggestion of the existence of this disease comes from a suspicious x-ray picture.

Confirmation may be made by sternal puncture. Rosenthal and Vogel⁴ state, "A definite diagnosis of multiple myeloma is possible during life only when made by means of sternal biopsy or the more simple aspiration method of Arinkin." Beizer et al.⁵ studied ten cases. In four cases backache was the

*See pages 65 ff.

¹Ewing, James: *Neoplastic Diseases*, ed. 4, W. B. Saunders Co., Philadelphia, 1940, p. 327.

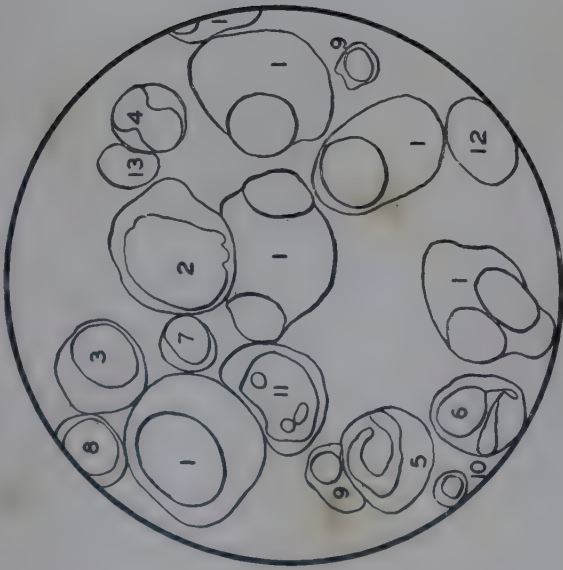
²Geschickter, C. F., and Copeland, M. M.: *Am. J. Cancer* 26: 441, 1936.

³Beizer, L. H., Hall, B. E., and Giffin, H. Z.: *Am. J. M. Sc.* 203: 829, 1942.

⁴Rosenthal, N., and Vogel, P.: *J. Mt. Sinai Hosp.* 4: 1001, 1938.

⁵Loc. cit.

PLASMA CELL MYELOMA



BONE MARROW
WRIGHT-GIEMSA STAIN X1,000

1. Large plasma cells, two with double nuclei
2. Degenerated promyelocyte
3. Neutrophilic myelocyte
4. Neutrophilic juvenile
5. Neutrophilic "stab"
6. Eosinophile
7. Small lymphocyte
8. Polychromatic macroblast
9. Polychromatic normoblast
10. Orthochromatic normoblast
11. Myeloblast
12. Polychromatic megalocyte
13. Polychromatic normocyte

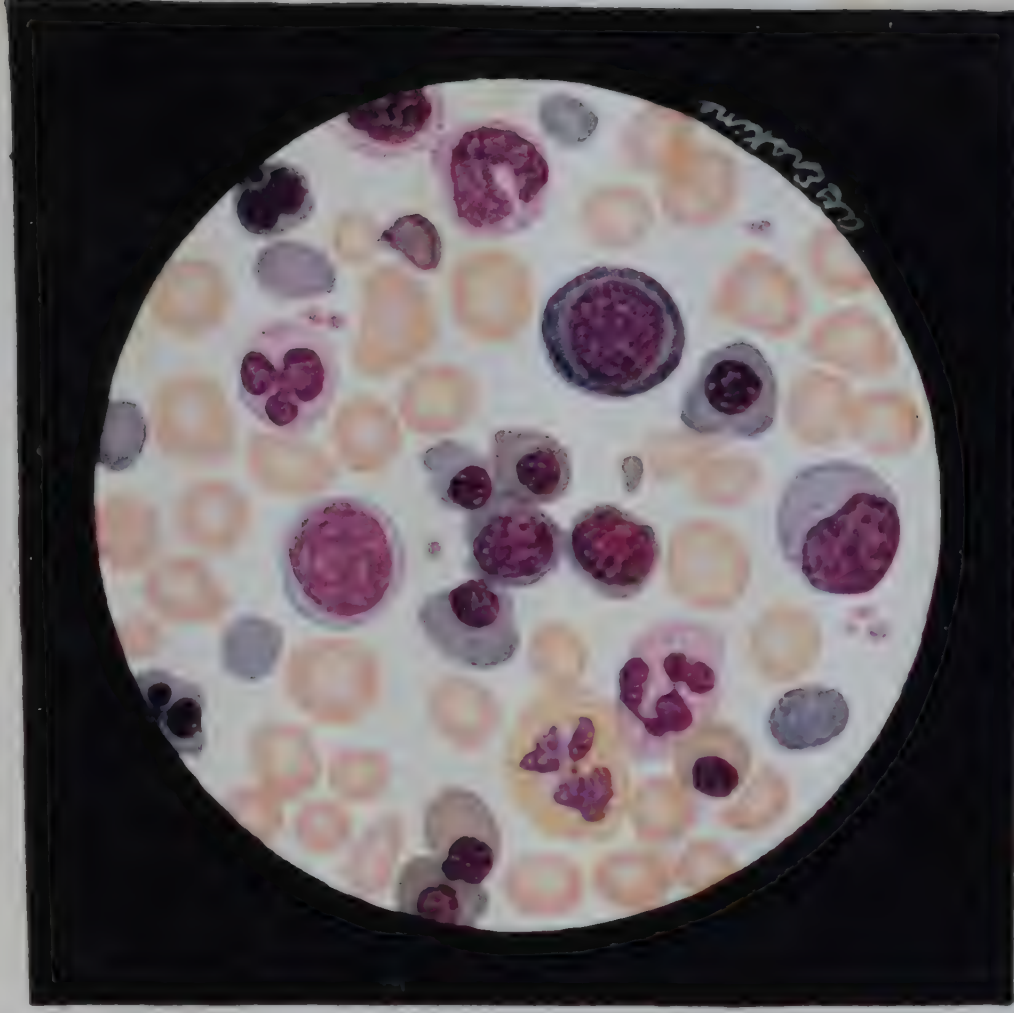
ERYTHROBLASTOSIS FETALIS



BLOOD FILM
GIEMSA STAIN X950

1. Normoblast in karyorrhexis
 2. Polychromatic normoblast
 3. Orthochromatic normoblast
 4. Polychromatic macroblast
 5. Polychromatic megaloblast
 6. Lymphocyte
 7. Segmented neutrophile
 8. Eosinophile
 9. Neutrophilic juvenile
 10. Polychromatic erythrocyte
 11. Polychromatic erythrocyte with Howell Jolly and Cabot ring bodies
 12. Blood platelets
 13. Neutrophilic "stab,"
- Note anisocytosis with macrocytes

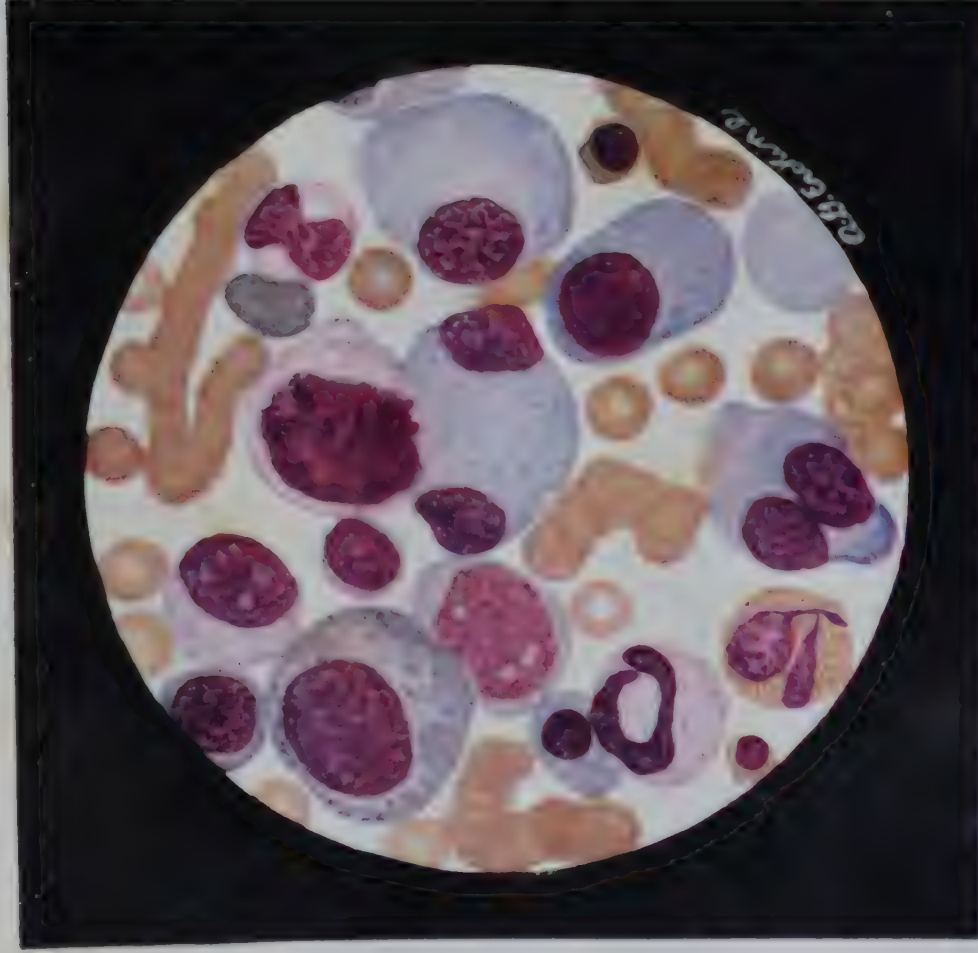
ERYTHROBLASTOSIS FETALIS



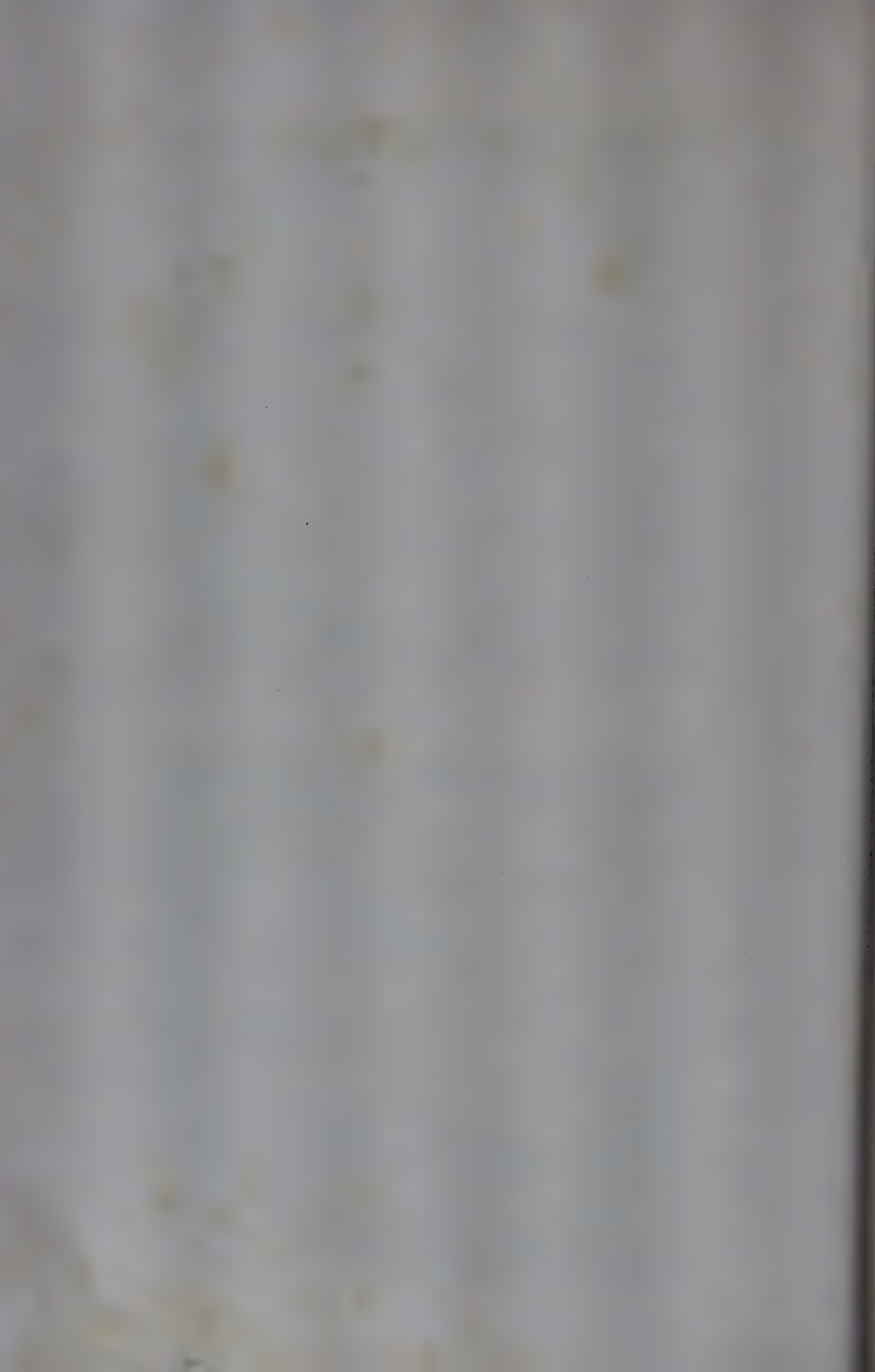
BLOOD FILM
GIEMSA STAIN X950

PLATE XXVII.

PLASMA CELL MYELOMA



BONE MARROW
WRIGHT - GIEMSA STAIN X1000



principal symptom. In one case, cervical pain was present and in another case arthralgia. Three cases sought relief of an existing anemia. In seven of the ten cases, x-ray pictures suggested the diagnosis.

A modified Arinkin method was used by these workers. From one to one and one-half cubic centimeters were aspirated from the sternal marrow into a paraffin-lined test tube containing a small amount of heparin. Spreads were stained by Wright followed by Giemsa stain. Diagnosis is confirmed by finding typical myeloma cells. An increase in the typical and atypical plasma cells is also suggestive of multiple myeloma. However, these investigators very properly caution that an absence of these findings in sternal marrow does not rule out the diagnosis.

Myeloma cells show an abundant, definitely outlined and deeply basophilic cytoplasm, a moderately sized round, excentrically placed nucleus having a fairly coarse chromatin sharply demarcated from the parachromatin, and a very large nucleolus.

Beizer calls attention to the fact that minor variations from this description may exist. The amount of cytoplasm varies from a narrow rim about the nucleus to a large quantity in others. Multiple small vacuoles may occur. A perinuclear zone may be seen. In one case they found 27.5 per cent plasma cells. These plasma cells had an excentrically placed nucleus containing coarse clumps of chromatin, and a few cells with somewhat larger nuclei, but no nucleoli, were seen. They emphasized the fact that the myeloma cell shows a large nucleolus and that in examinations of over 160 bone marrows no such cell was ever found in connection with any other pathologic process. Our own experiences convince us of the accuracy of Beizer's observations as to the specificity of the myeloma cells and also as to the occurrence of many plasma cells in this condition (see Plate XXVII).

Bayrd and Heck¹ mentioned that a negative diagnosis resulting from sternal aspiration is not always adequate evidence for excluding the possibility of multiple myeloma. Changes in the bone marrow may be irregularly distributed rather than diffuse, and the results on repeated aspirations are not uniform. In one instance in their experience a third aspiration of marrow was necessary to secure a diagnostic specimen.

So far as myeloma cells or atypical plasma cells are concerned, Osgood and Hunter² found 45 to 54 per cent of plasma cells in one case of a 49-year-old man. Osgood and Hunter considered this to be a true instance of plasma cell leukemia and not multiple myeloma with myeloma cells in the peripheral blood. They held splenomegaly, epistaxis, bleeding from the gums, and the clinical course which this patient pursued to be of the type characteristic of leukemia. As further evidence they cited the lack of Bence-Jones proteinuria, of "typical" defects of the bone in the roentgenograms, bone pain, and bone fragility. Others have felt that this is a distinction of degree only, not of kind. With this opinion Bayrd and Heck concur.

Meyer, Halpern, and Ogden³ reviewed all the known cases of multiple myeloma in which there were plasma cells in the peripheral blood and all the

¹Bayrd, E. D., and Heck, F. J.: *J. A. M. A.* 133: 3, 147-157, Jan. 18, 1947.

²Osgood, E. E., and Hunter, W. C.: *Folia haemat.* 52: 369-383, Nov. 1934.

³Meyer, L. M., Halpern, J., and Ogden, F. N.: *Ann. Int. Med.* 22: 585-598, April, 1945.

purported cases of plasma cell leukemia, of which they listed four. In a careful consideration of these cases they expressed doubt that any were actually cases of true plasma cell leukemia. They rejected these cases largely on the ground that the absence of osseous lesions had not been adequately established. They did report one case which they considered to be a true case of acute plasma cell leukemia, although they considered it to "complete the chain in the analogy to lymphoid tumors" such as lymphoma and lymphocytic leukemia.

Diagnosis of Multiple Myeloma.—The various clinical and laboratory features of this disease are striking and the occurrence of multiple osteolytic lesions of the bone, Bence-Jones proteinuria, and hyperproteinemia is strongly suggestive evidence for the presence of multiple myeloma. However, as pointed out by Bayrd and Heck, every effort should be made to confirm this with pathologic material for the sake of accuracy of prognosis and suggestions as to an outline of treatment. To this end, because of its simplicity and broad diagnostic range, the method of sternal aspiration readily lends itself.

Diagnosis of multiple myeloma can often be made by bone marrow aspiration. In this disease, the normal bone marrow elements are replaced by large numbers of plasma cells. When the percentage of plasma cells is quite high (40 to 90 per cent), the diagnosis is relatively simple. However, there are many instances in which lower incidences of bone marrow plasmacytosis (up to 20 per cent) may leave some doubt as to the diagnosis. We have had an experience corroborating that of others¹ that an increase in plasma cells may occur in a large heterogeneous group of chronic diseases, such as pulmonary tuberculosis, malignancy (with or without marrow invasion), rheumatoid arthritis, syphilis, sarcoidosis, cirrhosis of the liver, gold intoxication, Banti's syndrome, bacterial endocarditis, periarteritis nodosum, myelofibrosis, chronic leukemias (especially when irradiated), and diseases either members of or simulating collagen disease. A rather interesting feature of many of these cases is that they display disturbances of blood protein relationships, often showing elevations of the globulin fraction. In none of these secondary plasmacytoses have we seen the very abnormal plasma cells found in most cases of multiple myeloma. These "myeloma cells," as they are referred to by some observers, are larger than normal plasma cells, ranging from 15 to 18 μ in diameter, and have a slightly pinkish cast to the cytoplasm. However, other cases of myeloma have plasma cells indistinguishable from normal plasma cells. In general, abnormal forms with two to six lobed nuclei are much more common in myeloma than in the reactive plasmacytoses, although we have occasionally found trilobed plasma cells in the latter. Mitoses may be quite common in myeloma and are often abnormal in configuration, as is frequently true in many malignant diseases. Sections of the bone marrow are helpful in isolated cases of multiple myeloma of the nodular type. In such instances, spreads might not show an increase in plasma cells but the sections may show solid nodules of myeloma cells. In other instances the marrow may be exceedingly fatty and the spreads give an impression of extreme hypoplasia with very few cells seen. The sections in such cases show the true character of the disease.

¹Klein, H., and Block, M.: *Blood* 8: 1034, 1953.

The Bone Marrow in Hodgkin's Disease

Hodgkin's disease has a fairly high incidence of marrow involvement in studies where extensive autopsy search has been performed. However, in routine bone marrow aspirations, the yield is disappointing. An increase of reticuloendothelial cells with an eosinophilia is suggestive. Rarely, sections of the marrow will reveal the typical Hodgkin's nodule. Out of 46 proved cases of Hodgkin's disease, in only 4 cases were Hodgkin's nodules found and in all these instances serial sections of the marrow clot were searched vigorously. As with other marrow lesions with a spotty distribution, the success in finding such lesions is dependent in great part on the multiple sites and the search of many sections.

The Bone Marrow in Leukemia

Leukemia in almost all forms can be readily diagnosed by study of the peripheral blood. The most common exception to this is the acute stem cell leukemia with a low white blood count. We have found such cases in all age groups from 2 to 84 years, but have seen it most frequently in the third and fourth decades of life. Organ enlargements, such as splenomegaly, hepatomegaly, and lymphadenopathy, are frequently absent or tend to be minimal. Anemia is usually quite obvious clinically and the erythrocyte counts are down to 1,500,000 or 2,000,000 red cells per cu. mm. with correspondingly low hemoglobin values. Hemorrhagic manifestations are a frequent accompaniment of the disease and reflect a striking thrombopenia. Leukocyte values have ranged from 400 to 2,000 per cu. mm. In all these cases we have been able to find leukemic cells in the peripheral blood, with varying effort required in individual instances. In some of these the leukemic cells have been only a small percentage of the cells in the peripheral blood. The diagnosis of leukemia could be made on peripheral blood studies alone. However, bone marrow aspiration readily corroborated our impression. In all these cases the marrow was exceedingly hypercellular with an almost complete replacement of normal marrow elements by the leukemic cells. The striking difference between the cellularity of the bone marrow and the peripheral leukopenia in this condition raises a basic question as to what factors determine the delivery of cells from the marrow to the circulation. One would certainly expect a tremendous leukocytosis in this condition. Perhaps peripheral destruction due to unknown immunohematologic factors plays a role in the leukopenia, anemia, and thrombopenia.

In the chronic leukemias, there is fairly good correspondence between the peripheral blood changes and the bone marrow picture. The marrow tends to be hyperplastic, with the increase in cells corresponding with the cell type in the peripheral blood. The degree of shift to younger forms is inclined to be more pronounced in the bone marrow than in the peripheral blood. It is not too unusual to see this disparity in chronic myelocytic leukemia, where only a few myeloblasts are present in the peripheral blood but the bone marrow may show many such cells. In chronic lymphocytic leukemia, approximately 85 per cent of our cases showed typical leukemic hyperplasia. In the other 15 per cent, lymphocytic increases were noted but the marrow was not completely replaced by abnormal lymphocytes. Cases of chronic lymphocytic

leukemia, which were effectively treated with x-ray therapy, often showed so few lymphocytes in the bone marrow as to make the diagnosis of leukemia impossible. This was only rarely true in chronic myelocytic leukemia where, even though the marrow became less hyperplastic, the "monstrosity forms" of leukemic development were qualitatively readily identifiable.

Monocytic leukemia, especially in the subleukemic and chronic forms, could usually be diagnosed by the presence of an increase in abnormal monocytes in the peripheral blood. However, our experience with such cases has been that it is not unusual to find only 12 to 25 per cent of such cells in the peripheral blood. When the increment of increase was in the lower ranges, there were occasions when some doubt was cast on the validity of the diagnosis of monocytic leukemia. In all these cases, the bone marrow showed a hyperplastic state due to an overabundance of abnormal monocytes.

The diagnosis of such rare entities as **erythroleukemia (di Guglielmo's disease)** cannot be made except with the aid of bone marrow aspiration. In the three cases which we have encountered, there was profound involvement of the erythrocytic series. In addition to hyperplasia of the bone marrow due to an increase in cells of this series, there were qualitative changes in the red cell development of extreme pathologic character. "Monstrosities" were numerous and consisted of abnormal sizes, shapes, and nuclear structures of the red cells. Some cells reached the giant proportions of 35 to 40 μ and had as many as 5 to 8 nuclei. There was marked asynchronism between cytoplasmic and nuclear development and mitotic figures were often abnormal.

The Bone Marrow in Pernicious Anemia

Bone marrow aspiration studies in the anemias are generally not very fruitful from a diagnostic standpoint. Perhaps they are of greatest assistance in the macrocytic anemias, with pernicious anemia the most outstanding example. In this condition the classical bone marrow picture can be seen under the following conditions: (1) a red blood cell count of 3,000,000 per cu. mm. or less, and (2) no erythrocyte maturation factor (liver, folic acid, or vitamin B₁₂) has been administered. When the red cell count is above 3,000,000 per cu. mm., the typical bone marrow picture rarely is present, and as the red cell count increases, there is less and less likelihood of finding a typical picture. We have encountered cases displaying the typical neurological syndrome of pernicious anemia with little or no anemia. Bone marrow aspirations in these cases have been of no help in diagnosis. Insofar as the effect of therapy is concerned, it is well to remember that even modest dosages of erythrocyte maturation factor will quickly alter the bone marrow picture so that within 24 to 48 hours after such therapy, it may be difficult to make a diagnosis of pernicious anemia. Whenever the diagnosis of pernicious anemia is even suspect, no liver or similar therapy should be administered before the marrow aspiration has been performed. Since such a diagnosis is so important in committing a patient to lifelong therapy, anything that interferes with an accurate diagnosis is to be avoided. Early diagnosis and adequate therapy are the only certain means of avoiding serious neurological complications. Suboptimal therapy may mask the hematologic picture while these neuro-

logical features stealthily impose themselves on the patient. Once they have firmly established themselves, even the most vigorous treatment may fail. These are but a few of the reasons for accurate diagnosis of pernicious anemia.

The bone marrow in pernicious anemia in relapse is markedly hyperplastic. When the marrow puncture is being performed, many cases display tenderness of the sternum. As the marrow material is removed, its hyperplastic character can be seen grossly. Spreads of the bone marrow readily reveal this hyperplasia, which is primarily erythrocytic. The myeloid:erythroid ratio is obviously altered, approaching a 1:1 ratio in most cases and even showing a 1:2 or 1:3 ratio in other instances. Erythropoiesis is of the "primitive" type, simulating that of the embryo in the prehepatic stage. There is a marked shift to younger forms with the megaloblast the stem cell from which the red cells originate. Not only is there seen a profusion of megaloblasts but there is also noted a marked increase in other young forms, such as basophilic normoblasts and polychromatophilic normoblasts. Pathologic erythropoiesis reaches its greatest proportions in the bone marrow in pernicious anemia, being approached only in the rare cases of di Guglielmo's disease or other leukemic disease. These pathologic changes affect the cytoplasm as well as the nuclei of the red cells and consist of the following changes:

(1) Abnormal cell size. The megaloblast itself begins as a "giant" form, usually measuring 20 to 24 μ in diameter. There is little wonder, then, that the offspring of this cell are large. This developmental defect accounts for the macrocytosis.

(2) Asynchronism of development. In normal cell development, cell size, cytoplasmic staining, and nuclear size keep pace in an orderly manner. In pernicious anemia, this synchronism disappears. Large cells with small nuclei are common. Many cells may display an extremely basophilic cytoplasm and have the nuclei of fully developed normoblasts.

(3) Karyorrhexis of nuclei. Fragmentation of nuclei at all stages of development is readily apparent. This may vary from simple binucleated cells to erythrocytes containing one large nucleus surrounded by single or multiple Howell-Jolly bodies. Other red cells may display Howell-Jolly bodies only.

(4) Karyolysis of nuclei. Whereas normal nucleated red cells show karyolysis of the nuclei by a diminution in size, the erythrocytes of pernicious anemia frequently display this phenomenon within the nucleus. As a result, the stained nucleus takes on a "soil erosion" appearance, showing alternating areas of well-stained chromatin alongside areas which do not stain at all or stain a reddish hue. Not only can this phenomenon be seen in sizeable nuclei, but it is often visible in Howell-Jolly bodies as well.

(5) Mitotic activity is exceedingly great and is abnormal. The abnormalities take on many bizarre features. It is not unusual to see one or two chromosomes off by themselves in one part of a cell away from the major mitotic figure. When the mitotic figure shows separation of the chromatin, one pole may show considerably more material than the other. Abnormal condensations of chromatin are often noted and lysis of the chromatin in mitotic figures (just as in the intact nuclei) is observable.

There is a feature of pernicious anemia bone marrow that is mentioned fairly frequently but is rarely stressed, even in hematology texts, and this relates to the abnormal leukocyte development in this disease. This pathologic myelopoiesis is as regularly occurring in pernicious anemia as are the red cell changes; and it disappears with specific therapy just as promptly as the abnormal erythropoiesis. The most striking abnormality is the finding of "giant" white cells, particularly notable at the promyelocyte, myelocyte, and juvenile stages, reaching a size of 20 to 22 μ in diameter. In many of these cells there often is an inordinately great amount of nuclear material, giving the impression that the cell has all that it can do to contain the nucleus. When such cells go on to full maturity, they typify the "pernicious anemia neutrophile" with its increased size and multilobulated nucleus. Mitoses in the myelocytic series are increased and occasionally are abnormal.

The only other macrocytic anemias that we have encountered which are indistinguishable from pernicious anemia have been observed in tropical and nontropical sprue and in a rare case of *Diphyllobothrium latum* infestation. In all other instances, the differential diagnosis has been relatively simple. Advanced liver disease, such as cirrhosis, is occasionally associated with a macrocytic anemia. In none of the cases we have studied have we seen the degree of pathologic erythropoiesis and myelopoiesis observed in pernicious anemia in relapse. Macrocytosis may be striking in such cases but the other pathologic features are lacking. This has been true in cases of carcinoma (especially of the stomach), pernicious-like anemia of pregnancy, and some refractory anemias.

The Bone Marrow in Anemias

Insofar as anemias, other than the macrocytic group, are concerned, bone marrow aspiration is rarely diagnostic. The anemias of chronic hemorrhage and of hemolytic disease often show a nonspecific normoblastic hyperplasia of varying degree. This can be demonstrated in both spreads and sections of marrow. Aspirations of bone marrow in these cases is at times helpful in eliminating other diagnoses but the positive aids in anemia of this group come chiefly from history, physical examination, and peripheral blood studies.

There is a group of anemias in which **sections** of bone marrow material have given us more information than do the **spreads** of the same material. These include the anemias associated with malnutrition, terminal nephritis, and advanced liver disease. In all these instances, the sections of marrow show a marked increase in fatty marrow which replaces normal marrow. The marrow hypoplasia may become so marked that only isolated islands of blood-forming tissue can be seen interspersed between large fat globules.

The Bone Marrow in Polycythemia

In polycythemia, whether secondary or primary, there is a hyperplasia of all marrow elements which can be quantitatively appraised better in sections than in spreads. Block and associates¹ have used the appearance of sections as a guide for therapy in polycythemia vera. If the marrow sections show marked hyperplasia, they recommend further active radiation therapy,

¹Block, M., Bethard, W., and Jacobson, L. O.: Proc. Central Soc. Clin. Invest. 27: 17, 1954.

regardless of the peripheral blood values. We have noted an interesting and undescribed phenomenon in polycythemia vera related to the megakaryocytes. These cells show an unusual degree of cytolysis with all the cytoplasm of almost every megakaryocyte showing platelet formation and leaving bare nuclei surrounded by platelets. This phenomenon might account for the thrombocytosis commonly associated with polycythemia vera and has not been observed in the secondary polycythemias.

The Bone Marrow in Malignant Lymphomata

Malignant lymphomata at times may be diagnosed by bone marrow aspiration. For this diagnosis we still prefer biopsy of lymph nodes over bone marrow aspiration because the former will yield a higher return than the latter. Table 76 requires a word of explanation. The 100 per cent diagnosis under sections as compared with only 9.4 per cent positives in spreads are comparative figures between the two technics. We have not found that 100 per cent of lymphosarcomata can be diagnosed by marrow aspiration but that when the diagnosis is made this way, the sections of marrow will show it ten times as frequently as the spreads. This disparity between the two technics is readily comprehensible when one observes numerous sections of such cases.

Lymphosarcoma has a tendency to form **nodules** in bone marrow rather than to be diffusely spread throughout the marrow. The usual picture is that of a central nodule, consisting solely of lymphosarcoma cells, within a marrow unit, the periphery of which consists of normal marrow elements. Not infrequently this peripheral zone shows a marked eosinophilia. Alongside such affected marrow units may be seen perfectly normal units and these generally outnumber the involved areas. In the preparation of spreads from such material, if one does not happen to catch a nodule of lymphosarcoma cells, the diagnosis will be missed. We have the impression that these lymphoid nodules are held together rather compactly and do not spread well. In the spreads in many of these cases, one may encounter a moderate increase in abnormal lymphocytes but only of sufficient degree to arouse suspicion of the diagnosis. A generalization that appears reasonable from our observations is that when there is an increase in abnormal lymphocytes in the peripheral blood, the bone marrow is likely to show a diffuse spread of these cells. This is the pattern of chronic and acute lymphocytic leukemia and of leukosarcoma. As a corollary to this observation, when we see the nodular type of lesion in the bone marrow, then abnormal lymphocytes are rarely seen in the peripheral blood. At times, we have observed one type transform into the other, most frequently the nodular type changing into the diffuse type of disease.

A word of caution is in order concerning lymphocytic nodules in bone marrow. Isolated nodules may be seen in normal bone marrows and in our experience are fairly regularly encountered in elderly subjects, particularly those with malnutrition. The finding of an isolated lymphocytic nodule, therefore, does not make a diagnosis of lymphosarcoma. We insist on the finding of goodly numbers of such nodules before making this diagnosis. In well-prepared thin sections, it is often possible to identify individual lymphocytes which are abnormal. An increased incidence of mitoses adds further weight to the diagnosis.

We have found bone marrow aspirations helpful in two situations where lymphosarcoma is suspect. By far the largest group has been those patients who have mediastinal or abdominal involvement and no readily accessible peripheral lymphadenopathy. Marrow aspiration in these cases is a much simpler approach to diagnosis than thoracotomy or abdominal exploration. The other situation in which we have found marrow aspiration useful is in determining the extent of involvement of the disease. We have observed patients with lymphosarcoma of the skin, with no significant organ enlargements or lymphadenopathy, who have shown extensive marrow involvement. In these cases we have recommended more vigorous therapy than would have been used for the skin lesions themselves. Prognostically, the cases with marrow involvement have had a poorer outlook than those without such spread of the disease.

Infections and Bone Marrow

The usual response of the bone marrow in acute infection is a moderate hyperplasia of the myelocytic elements with a left shift in this series paralleling the shift seen in the peripheral blood. At times this shift may reach the proportions seen in chronic myelocytic leukemia. These are several helpful differential points in the bone marrows of these two conditions, as listed in Table 78.

TABLE 78

	INFECTION	MYELOCYTIC LEUKEMIA
Toxic changes in leukocytes	Present	Absent
"Monstrosity" forms	Absent	Present
Increased basophiles	Absent	Often present
"Hiatus" phenomenon	Absent	Often present
Myeloblasts	Few	Usually plentiful

In the field of chronic infections with specific granuloma lesions, bone marrow studies may make the diagnosis. Brucellosis, tuberculosis, and histoplasmosis are examples of this group. The success in finding these lesions is dependent on the multiplicity of punctures and the vigor with which the search for them is conducted. Obviously, sections of the bone marrow are superior to spreads. Granuloma lesions, such as sarcoid, belong to the same category.

Spreads may be helpful in finding the organisms of leishmaniasis, histoplasmosis, and toxoplasmosis. Although articles have appeared suggesting bone marrow studies for malarial infestations, we have not found this procedure necessary. After an extensive experience with malaria in the Armed Forces, our recommendation in this search for malaria is thorough knowledge of the thick drop technic. We were not able to find a single instance of malaria in which we could demonstrate the parasites in bone marrow material and not find them as readily in thick drop preparations.

Culture of bone marrow for isolation of certain organisms has been successful in isolated instances, such as brucellosis and tuberculosis.

The Bone Marrow in Aplastic Anemia and Myelofibrosis

In these states the bone marrow may become exceedingly hypocellular or almost completely replaced by fibrous connective tissue. Some comment has already been made concerning the relative merits of spreads and sections in these conditions, with the section technic holding the upper hand. If, however, successive "dry" taps are encountered, surgical trephine should be performed before making such a diagnosis.

In addition to the above-mentioned idiopathic state, we have encountered variable degrees of marrow fibrosis in other conditions. The chronic blood dyscrasias, such as leukemia and polycythemia, which have received significant amounts of radiation therapy may show a considerable degree of fibrosis. The same is true of Hodgkin's disease treated with radiation or nitrogen mustards. Some cases of metastatic carcinoma show extensive fibrous replacement of bone marrow. We have observed this to be particularly true in cases where we were able to demonstrate capillaries plugged with tumor cells. In most of our cases of metastatic carcinoma, this was not true; normal marrow units were seen alongside large aggregates of tumor cells. A few cases of sarcoidosis and a rare case of widespread tuberculosis showed marked replacement of normal marrow.

Contraindications to Marrow Aspiration

Bone marrow aspiration is not indicated until adequately complete history, physical examination, and peripheral blood studies have been performed and indicate the desirability of the procedure. In addition to the discomfort to the patient, the procedure carries some danger, a few deaths having been reported as a result of bone marrow aspirations. Perhaps the only medical contraindication to marrow aspiration is hemophilia, in which uncontrollable bleeding may follow the puncture.

The Technic of Bone Marrow Investigations

The first instrument for the examination of bone marrow was made by Wolff¹ in 1903. This examination was performed by trephination of the diaphyses of the tibias of small animals. Human bone marrow of a living subject was first examined by Ghedini² in 1908. This method, following the method of Seyfarth, was used by Schilling and this author (R. B. H. G.) for some time, but has been superseded by the relatively simple process of direct sternal puncture instead of trephining as we previously performed this technic. The difficulties incident to an operation of the type of trephination were removed by Arinkin³ in 1929. He used a large needle and trocar and punctured the body of the sternum under aseptic conditions, using a local anesthetic.

Gradwohl⁴ reported a new instrumentarium for performing sternal puncture. It consists of a 10 c.c. Luer Lok syringe, a cannula and connector with a rubber tubing joint, a bone marrow puncturing instrument with a guard which can be adjusted to fit the depth of the sternal puncture, and an obturator for the latter.

¹Wolff, A.: *Deutsche med. Wchnschr.* 29: 165, 1903.

²Ghedini: *Wien. klin. Wchnschr.* 23: 1840, 1910.

³Arinkin: *Folin haemat.* 38: 233 (June), 1929.

⁴Gradwohl, R. B. H.: *J. A. M. A.* 108: 803, 1937.

The skin over the sternomanubrial junction is surgically prepared, the usual sterile drapes are applied, and local anesthesia with procaine hydrochloride solution is injected intradermally, subcutaneously, and into the periosteum. The instrument for puncture has a handle which permits a firm grasp and allows one to bore through the external lamina of the sternum and enter rapidly the marrow cavity of this bone. The instrument is thrust through the skin at the sternomanubrial junction at right angles to the surface of the sternum. The guard on the shaft of the needle is so adjusted that the instrument



Fig. 220.—The Gradwohl-Aloe sternal marrow puncture apparatus: *A*, syringe; *B*, cannula and connecting tubing; *C*, bone marrow puncture instrument; *D*, obturator.



Fig. 221.—Bone marrow touch preparation.

cannot possibly be pushed through the sternum into the mediastinal space. After the instrument has well penetrated the sternum, the direction is changed with the point directed upward. At this time, when the marrow has been entered, the obturator is removed, the connector and syringe are attached above the handle of the boring instrument, and suction is applied. One obtains in this way a very definite specimen of the bone marrow. It goes without saying, of course, that the entire instrumentarium must be adequately sterilized before use. One may place a small amount of dilute sterile sodium citrate solution in the barrel of the syringe, which slightly dilutes the bone marrow material.

The large caliber of the instrument permits one easily to obtain sufficient bone marrow. Once the marrow is obtained, the instrument is rapidly removed and the opening closed with a surgical dressing. Repeated punctures on the same patient can be made with this instrument without any difficulty.

It is not necessary to remove a large quantity of bone marrow. Only a small amount is obtained, usually about 0.2 to 0.3 c.c.

Fig. 220 shows the entire set-up for the procedure.

Turkel Technic for Biopsies of Sternum

The Turkel trephine biopsy method eliminates the two common objections to marrow biopsies—(1) that biopsy is a surgical procedure, and (2) that biopsy leaves a disfiguring scar.

The advantages of this method are that it is easily and rapidly performed at bedside or office and does not leave a disfiguring scar. It controls the depth and force of penetration; in other words, it prevents penetration of posterior lamella and mediastinum. There is no dilution of marrow with circulating blood. There is preservation of marrow topography. The biopsy by this method shows cells adherent to the marrow wall which are not seen by aspiration. Further, the biopsy shows the true proportion of cells in marrow, or the myeloid-erythroblast ratio. It checks results of treatments quantitatively. It possesses the advantage that aspiration and infusion may be performed through the same aperture.

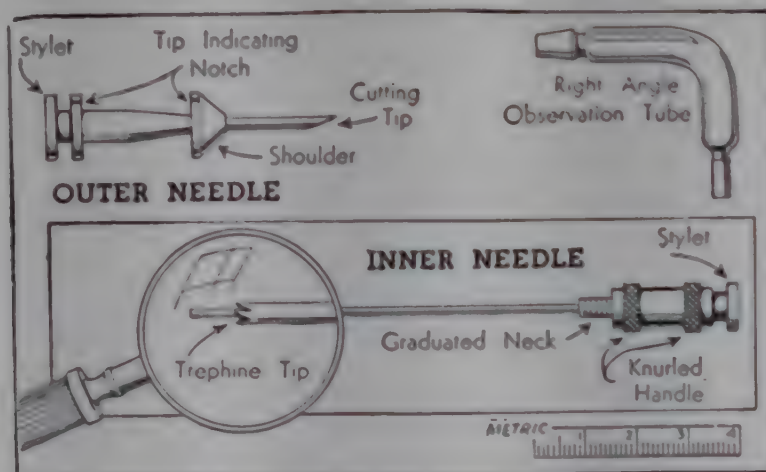


Fig. 222.—The Turkel needle for aspiration or biopsy of the sternum.

Technic.—

Cleanse the skin over the desired site, usually opposite the third rib, with surgical antiseptic. Infiltrate skin, subcutaneous tissue, and periosteum with local anesthetic.

Insert outer needle with styler in place (Fig. 222) with the tip of the needle in the direction of the face of the patient at an angle of about 45 degrees, through the skin, subcutaneous tissue, and periosteum, until the tip just engages the anterior lamella of the sternum.

Leave the outer needle in place and remove the styler.

Insert the trephine needle with styler into the outer needle.

Remove styler of inner trephine needle.

While holding the outer needle with the fingers of the left hand, turn the head of the trephine needle with the fingers of the right hand, with a slight clockwise-counterclockwise motion, at the same time exerting gentle inward pressure. A sudden release of resistance signifies entrance of the trephine tip into the sternal cavity. The clockwise-counterclockwise

motion, with slight pressure, should continue until the neck of the inner needle is completely within the head of the outer needle. Rotate the head of the inner needle in order to detach the plug from the surrounding marrow.

(a) **For Aspiration.**—While holding the knurled handle of the inner needle, push the outer needle into the sternum about 6 mm., until the neck of the inner needle becomes completely visible. Remove the inner needle. Attach the syringe, aspirate about 1 c.c. of blood-marrow mixture. Remove syringe, express mixture immediately into oxalated tube.

(b) **For Biopsy.**—Remove the inner needle and with its stylet force the marrow plug into a bottle containing a fixative or use it as imprint or spread.

Insert stylet into outer needle, remove outer needle, and cover the point of insertion with antiseptic dressing.

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Marrow Biopsy, Tibia

In infants and younger children it is safer to utilize the tibia instead of the sternum in obtaining bone marrow specimens. Except for the location, the technique is the same as that used in sternal puncture.

Use of Iliac Crest in Bone Marrow Punctures*

Bone marrow can be obtained easily and safely from the iliac crest and may at times provide information not obtainable from sternal aspiration.

Technic.—

The site for the iliac crest puncture is chosen on the crest over an area within 3 cm. posterior to the anterior superior iliac spine on either side of the body. According to the thickness of the tegument a needle 1 inch to 2½ inches long, 16 gauge, and furnished with a stylet, is used for puncture; a 20 cm. tightly fitted dry syringe is used for aspiration.

The determined site is prepared with iodine and alcohol and infiltrated with 2 per cent Novocain solution until the periosteum is reached; the periosteal space is then injected very slowly; usually no more than 2 c.c. of the Novocain solution are required. The patient lies in the supine position; in cases of very pronounced abdominal distention (ascites, enlarged viscera) the patient is tilted on his side. The puncture is performed with the needle held at a 45 degree angle to the long axis of the body and with pressure applied by the palm of the hand. The aspirating needle is forced into the bone with steady pressure and slight rotation. A distinct "give" sensation is usually felt when the needle enters the medullary cavity. As the needle is firmly embedded in the medullary cavity the stylet is withdrawn and the syringe attached to the needle. In order to avoid admixture of blood the plunger is slowly withdrawn until the first drop of marrow fluid is observed in the capillary end of the syringe. Bone marrow aspiration is accompanied by a slight sensation of pain (suction pain).

The material removed may be used in the usual way for enumeration of total nucleated cell count, for preparation of smears, and for section study.

The technical advantages of iliac crest versus sternal puncture are:

1. No injury can be sustained by any underlying organ.
2. Usually the iliac puncture is a less painful procedure and the patient is less apprehensive than when subjected to the puncture in the cardiac area.

*Michael A. Rubinstein, Montefiore Hospital, New York, N. Y. Personal communication based upon facts by Dr. Rubinstein in his Scientific Exhibit, A. M. A. convention, Atlantic City, N. J., June, 1947.

3. Repeated aspirations of the iliac crest can be easily performed at frequent intervals on both sides; iliac aspiration is very suitable for serial bone marrow studies. This may be important especially in experimental pharmacologic physiologic research of hematologic tissue and bone marrow tumors.

It is suggested that the marrow cavities of the iliac bone can be used as a readily available route of administering fluids into the general circulation when no intravenous route is accessible and they allow for greater safety than the sternal technic.

Disadvantage of the iliac aspiration is the occasional difficulty of puncturing the bone which at times may prove to be exceedingly firm in this region.

Bone marrow studies were performed simultaneously in iliac crest and sternal aspiration in 216 different cases showing normal and pathological findings.

Normal Values.—The normal values of the iliac bone marrow were found to be approximately in the same range as those determined for sternal aspiration. Iliac crest counts in hematologically active bone marrow in all age groups of normal individuals studied (age range 16 to 78 years) gave the following results: The total nucleated cell count of the iliac bone marrow, as that of sternal aspiration, tends to decrease with age from the average of 110,000 per cu. mm. in persons under twenty to 50,000 per cu. mm. in those over sixty. The differential count of the normal iliac marrow ranged as follows: myeloblasts, 0.5 to 2%; promyelocytes, 1 to 5%; myelocytes, neutrophilic, 11 to 23%; myelocytes, eosinophilic, 1 to 3%; myelocytes, basophilic, 0.5 to 1%; nonsegmented neutrophiles, 8 to 29%; segmented neutrophiles, 5 to 15%; eosinophiles, 1 to 3%; basophiles, 0.5 to 2%; lymphocytes, 2 to 10%; plasma cells, 0.5 to 3%; reticulum cells, 0.5 to 2%; hematogones, 0 to 3%; proerythroblasts, 0.5 to 1%; erythroblasts, 2 to 8%; normoblasts, 10 to 21%. The number of megakaryocytes ranged between 22 and 88 in 1 c.mm.

Examination of Bone Marrow Preparations

Touch preparations are made on the surfaces of clean, fat-free glass slides. A few spreads also are made. See method of Agress, page 943.

The combination of Wright and Giemsa stain is the best staining method. The specimens are dried in the air and stained with Wright stain 1 minute followed by differentiation in neutral distilled water for 5 minutes and then washed. (Carry out the usual Wright staining procedure, page 620.) The specimens are then stained for 30 minutes with a dilute Giemsa stain made by adding a drop of stock Giemsa stain to each c.c. of neutral distilled water.

After the staining process is completed the slide is washed with neutral distilled water, dried in the air, and examined. This combination stain gives excellent results.

In connection with the technic of bone marrow investigations, which is described above, a communication by Isaacs¹ outlines his method of counting the cells of the bone marrow which is of inestimable benefit in the determination of the type of anemia. The description is as follows:

Wet the inside of the tube of a Sahli hemoglobin pipette, or the stem of a red or white blood cell counting pipette with serum. The pipette stem should have markings enabling one to measure 4 volumes. A serum of the same blood group as the patient is desirable if the red blood cell count is a factor, otherwise any blood serum may be used. The serum is drained out, and bone marrow is sucked up 1 unit of space into the pipette. By working the tip back and forth among the trabeculae of bone, enough marrow may be obtained without air bubbles. Serum is then drawn up so that the total bulk is 4 volumes. This is then discharged into a watch glass, using care to wash out all of the marrow. Eight more volumes of serum (measured by the markings on the pipette) are then added, and the suspension is stirred thoroughly with a blunt glass rod. This suspension is then drawn into a red blood cell counting pipette to the 0.5 mark, and diluted with Hayem's solution, or with 1% acetic acid if a count of leukocytes only is desired, the rest of the steps being exactly as in the case of counting red blood cells, using a hemacytometer slide. The high-dry objective is used. The

¹Isaacs, Raphael: *Am. J. M. Sc.* 193: 181-191, February, 1937.

total number of cells in 4 or 8 sq. mm. are counted, the adult red blood cells being enumerated in one group (if acetic acid has not been used) so that when their number is subtracted from the total, the number of nucleated cells of all types is obtained. The calculations are as follows:

The number of cells counted in 1 sq. mm., obtained by dividing the total number of cells counted by the number of square millimeter spaces used, is multiplied by 24,000; i.e.,

$$\frac{10 \times 12 \times 200}{\text{No. of sq. mm.}}$$

The differential count is made from cover glass films, using a thick emulsion of bone marrow in serum. The fresher the serum, the better are the results. If the serum is of the same blood group, the red blood cells show a better distribution than when they are agglutinated. Human serum may be used for bone marrow of animals. Use Wright or Giemsa stain. When Wright stain is used, allow the stain to act for 1 minute, then dilute with as much distilled water as the cover glass will hold, mixing very thoroughly. Allow this to stand for 10 minutes, then wash with distilled water, dry and mount in xylol-balsam.

The most "representative" bone marrow is obtained from the midsternum, as this gives both red and white cells in the course of their development. This technic may be used with marrow from any bone or for cells of other organs.

With careful technic, the variation in counting cells from the same region in the normal marrow, using several pipettes, averages about 5%, an amount but slightly greater than variations from drop to drop in the same pipette. In pathologic marrows, where there was a grossly uneven distribution of the cells, the variation was considerably greater, amounting to as much as 24% between a cellular and relatively noncellular region, and to 85% in marrows with regions of "aplasia."

With this method, Isaacs maintains that there are from 150,000 to 300,000 cells per cu. mm. in normal sternal bone marrow. He states that the "primitive blast" stage of the cells of the polymorphonuclear leukocyte series resembles that of the lymphocyte and erythrocyte series of cells in general morphology, and may or may not be identical. The next stage in the development of the red blood cell he terms the megaloblast, and believes that this is a normal stage in the production of the erythrocyte rather than a pathologic or fetal cell, as many other authorities believe. He calls the next stage in development the basophilic normoblast or macronormoblast. Isaacs calls attention to the variation in the number of cells in the bone marrow dependent upon changes in physiology. In other words, a normal individual may tax his granulopoietic tissue at one time more than another in trying to remain normal. His view of the normal composition of the cells of bone marrow follows:

	PER CENT OF ALL NUCLEATED CELLS
Undifferentiated "lymphoid" cells. "Primitive blasts" -----	23.1 ± 8.0
Megaloblasts -----	3.0 ± 1.0
Basophilic normoblasts -----	7.2 ± 2.5
Eosinophilic normoblasts -----	12.0 ± 7.0
Polymorphonuclear neutrophiles—adult -----	7.5 ± 5.5
Polymorphonuclear neutrophiles—young -----	13.0 ± 6.0
Metamyelocytes -----	20.8 ± 3.0
Myelocytes -----	2.8 ± 1.8
Myeloblasts -----	1.8 ± 0.8
Eosinophiles (immature and mature) -----	5.5 ± 3.0
Lymphocytes -----	1.0 ± 0.5
"Endothelial" cells. Phagocytes -----	0.9 ± 0.5
Hemohistioblasts and hemohistiocytes -----	2.3 ± 1.3
Megakaryocytes -----	0.5 ± 0.1
Basophiles -----	Present
Monocytes -----	Present

He calls attention to the fact that in aplastic anemia the total number of cells per cubic centimeter is greatly reduced and the fat content is increased. So-called idiopathic aplastic anemia differs from aplastic anemia following intensive roentgen ray therapy in that in the latter condition the number of primitive blasts is reduced, but the cells which are present tend to ripen into eosinophilic normoblasts and later into mature erythrocytes. In the hypoplastic anemia of nephritis the inhibition is just after the primitive blast stage, so that cells in this stage accumulate in normal or greater than normal numbers while but few ripen to the megaloblast stage.

In pernicious anemia, he found that the point of inhibition appears at the megaloblast stage. Inhibition appeared at the normoblast stage in leukemia and infection.

The next stages are eosinophilic normoblast, reticulocyte, polychromatic red blood cell, and the mature erythrocyte.

Agress Modification for Preparing Spreads and Sections Simultaneously

Perform marrow aspiration in the standard manner, using the site of the operator's choice or as indicated in individual problems.

Attach a *dry*, sterile, tight-fitting 5 or 10 c.c. syringe to the aspirating needle.

Make a *strong, sudden* pull with the plunger, withdrawing only about 0.5 to 1.0 c.c. of marrow. This first show of marrow generally is the most cellular and has the least dilution with blood.

Quickly transfer the entire contents of the syringe to a clean glass slide.

Touch this pool of marrow by the edges of successive slides and then spread over individual slides to make the spreads.

Generally 8 to 12 spreads should be made of each preparation.

Stain spreads as outlined on page 941.

When the pool of marrow has clotted, pick up the clot with the stylus of the aspirating needle, and transfer to a fixative. Thereafter treat like a routine surgical biopsy. Our preference in fixatives is Zenker's acetic acid (10%), which permits the use of special stains if desirable. The routine staining is hematoxylin and eosin, with particular care exercised in differentiation.

Using this method, Agress has found that 92 per cent of his spreads and 95 per cent of his sections are satisfactory.

Anticoagulants are *not used* at any time in the routine preparation of bone marrow spreads or sections. However, lupus erythematosus ("L.E.") cells will only rarely be found in using this technic. If L.E. cell preparations are desired, the method differs.

For L.E. Cells.—

Leave the aspirating needle in situ.

Aspirate 1 to 3 c.c. of marrow into a syringe containing a few drops of heparin or other anticoagulant.

Allow this material to incubate at 37° C. for 2 hours.

Centrifuge at 1,000 r.p.m. for 5 minutes.

Make spreads of the "buffy" coat, and stain in the routine manner. The method of staining is given on page 941.

For Cultures of Bone Marrow.—

If cultures of bone marrow are desirable, these, too, are collected after the routine method has been performed, leaving the aspirating needle in place. A clean, sterile syringe, containing sterile anticoagulant, is reapplied, and an effort is made to obtain as much as 5 c.c. of marrow material. This is then transferred to appropriate media.

Culture of Human Bone Marrow

Osgood has carried out some very interesting research work on cultivation of bone marrow. He has presented this work in various communications.¹ This method was used by him for the appraisal of therapeutic agents. It permitted determination of their effect on living human cells, of the toxic dose, and the minimum therapeutic dose, as well as the accurate control of concentration. He showed by his culture method a number of points about the length of life of neutrophiles, eosinophiles, and basophiles of normal blood by comparing blood and sternal marrow from healthy persons. The cultures were made in 30 c.c. vaccine vials in a medium consisting of about 35 per cent of human cord serum and 65 per cent of the balanced salt solution of the Geys.² This medium was previously determined by the Geys to be satisfactory for the culture of human tumors. Osgood found it possible to maintain cultures of human marrow in as nearly their natural state as any medium so far investigated. Note that the medium contains no substance not present in normal blood. The method by which he carried out this work is described by him in the *American Journal of the Medical Sciences*, February, 1938, vol. 195, pp. 141-144.

Method.—

Assemble the apparatus from 5 two-liter aspiratory bottles,* Pyrex standard wall glass tubing, and pressure tubing. Test its operation. Determine the volume of fluid in bottle 1 at the level of the inlet orifice of 4. Connections must be gas-tight under a pressure of at least 60 inches of water. Determine the number of drops per c.c. from tips 21 and 11. With water instead of medium flowing through the apparatus at the desired rate for use with the marrow, adjust the temperature of the water bath so that the temperature as determined on the fluid within 1 after a period of time for equilibrium to be reached is 37.5° C., the optimum temperature for the experiment. The temperature in the flask is usually about 1° lower than in the bath, but it should be determined for each experiment. Now disassemble the apparatus at convenient joints. Sterilize bottles 1, 5, and 9 with their connections as far as 23, in the autoclave, wrapping the free ends in paper or gauze. The gas system, including bottles 13 and 19 and their connections, need not be sterile. Reassemble the apparatus using aseptic technic, flaming the ends of glass tubes and pouring 70% alcohol on the rubber tubing before making each connection. Introduce the marrow, obtained as described by Osgood,³ into bottle 1 through vaccine cap 2.

Introduce medium³ with syringe and needle through rubber tube 7, previously sterilized with alcohol, into bottle 5. Close screw clamp 8. Force air in through 6 until the medium is all transferred to reservoir bottle. Used compressed air or a blood pressure manometer bulb. Disconnect bulb from 6 and adjust the flow of the medium from reservoir 9 by screw clamp 10, counting the number of drops per minute from tip 11, previously calibrated so that the c.c. per hour or 24 hours can be calculated. Any desired gas mixture can be made up in a spirometer and introduced into bottle 13 by closing screw clamps 14 and 15 and filling bottle 13 completely full with 5 to 10% sulphuric acid.⁴ Attach the outlet of the spirometer to inlet 16 and allow the gas to flow into bottle 13 by opening screw clamps 17 and 18, collecting the acid as it flows out in reservoir bottle 19, which is disconnected for this purpose.

*Any size container may be substituted for these without altering the principle of the method.

¹Osgood, E. E.: *J. A. M. A.* 109: 933-936, 1937. *Am. J. M. Sc.* 195: 141-144, 1938. *Am. J. Clin. Path.* 8: January, 1938.

²Gey, G. O., and Gey, M. K.: *Am. J. Cancer* 27: 45-76 (May), 1936.

³Osgood, E. E., and Brownlee, I. E.: *J. A. M. A.* 108: 1793, 1937.

⁴This prevents growth of molds and bacteria and dissolves less carbon dioxide than water.

When 13 is almost empty, close 18 and 17, place 19 back on its shelf, and attach to outlet 20. Open 14 and adjust the rate of flow of acid by the number of drops per minute of calibrated tip 21 to give the desired rate of gas flow. Open 15. Filter the gas leaving bottle 13 through sterile cotton 23, pass through trap 24; in passing down tube 3, it equilibrates with the medium flowing through the same tube as it enters culture bottle 1.

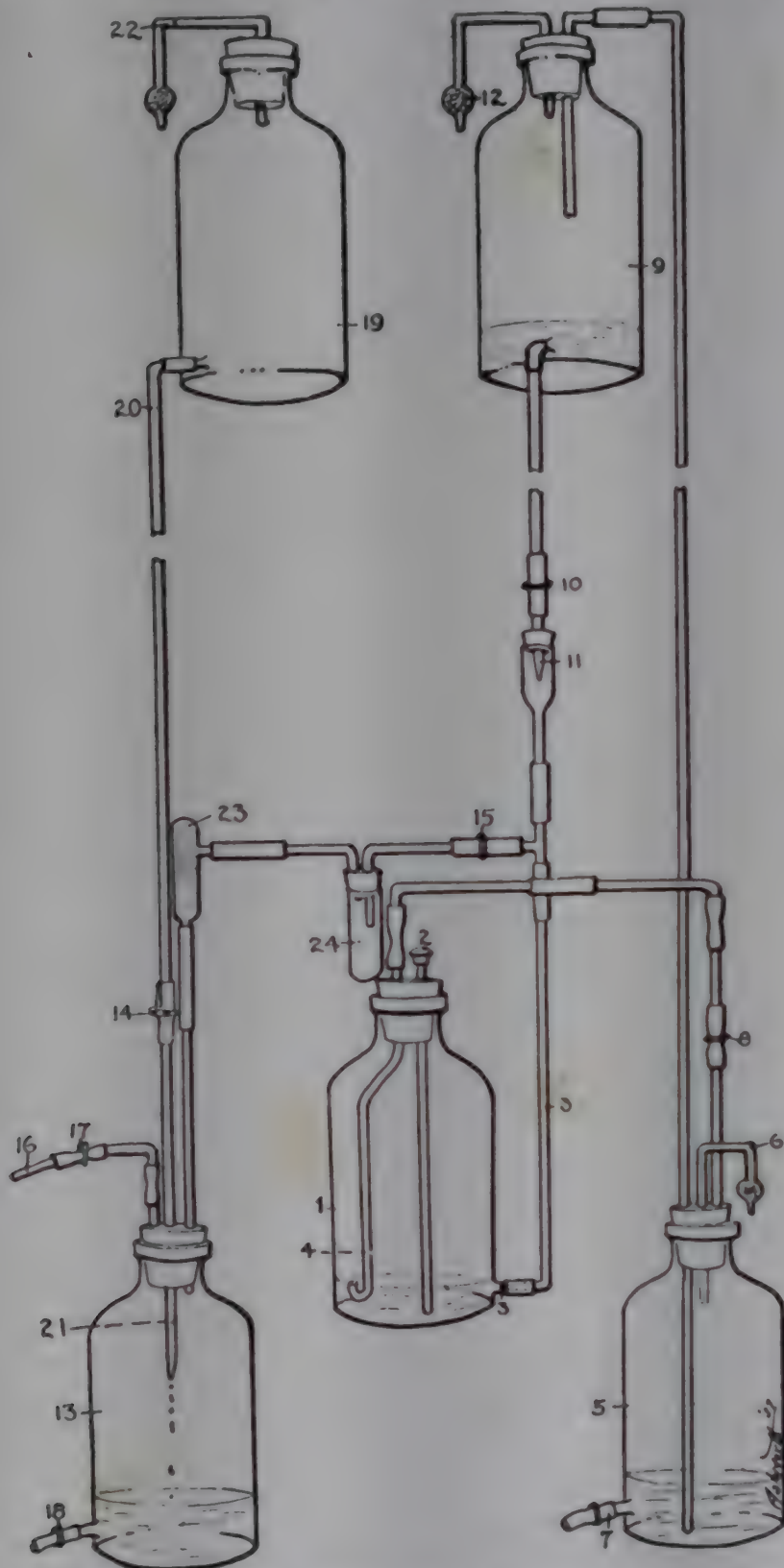


Fig. 223.—Osgood apparatus for bone marrow culture. (From Osgood: *Am. J. M. Sc.*, February, 1938.)

Medium¹ and gas enter through fine tip 3. The medium, being slightly cooler than that in the flask, flows across the bottom, and then gradually rises to the surface. Pressure of inflowing medium and gas forces the used medium and gas out through tube 4 into reservoir 5, from which the gas escapes through cotton-filled bulb 6. It may be collected if desired.

¹Osgood, E. E., and Brownlee, I. E.: *J. A. M. A.* 108: 1793, 1937.

If desired, tilt culture bottle 1 at any time so that the inlet of 4 is above the level of the liquid. Thoroughly mix by shaking, and aspirate marrow culture through 2 for counts and examination.¹ Calculate total numbers of any cell type present by multiplying the number per cubic millimeter by the volume of the culture in cubic millimeters. No semi-permeable membrane is necessary because the cells settle out and only the surface layer of fluid flows out through tube 4.

With this method, a rapid, controlled rate of flow of the medium past the cells and a rapid, controlled rate of gas supply is possible. Medium from bottles 1, 9, or 5 may be aspirated with a sterile syringe and needle through the pressure tubing at the outlet at any time for chemical, bacteriologic, or serologic examination. Medium may be used several times by pumping it as described from bottle 5 to bottle 9. Gas may be collected at 6 for analysis.

TABLE 79.—AZUROPHIL GRANULES

DIAMETER OF CELL IN RELATION TO LOBOCYTE	SIZE OF GRANULES	NUCLEOLI	CHROMATIN STRUCTURE	SHAPE OF NUCLEUS	PEROX-IDASE STAIN	NAME OF CELL
Same or smaller	Coarse	Present or absent	Coarse in clumps	Round or oval, sometimes irregular or cloverleaf	Negative	Lymphocyte
Larger	Coarse	Present or absent	Coarse	Round or oval, rarely irregular or cloverleaf	Negative	Prolymphocyte
				Round or oval, rarely horse-shoe	Positive	Progranulocyte A (Promyelocyte II)
			Very coarse	Round or oval	Negative	Plasmacyte†
			Fine	Round or oval, rarely horse-shoe*	Negative	Lymphoblast or granuloblast
	Fine, diffuse	Present	Fine	Round or oval Horseshoe or irregular	Negative Positive or negative	Monoblast Promonocyte
		Absent	Coarse clumps and strands	Horseshoe or irregular	Positive or negative	Monocyte
3 times as large	Fine, diffuse	Absent	Coarse clumps and strands	Horseshoe or irregular	Negative	Megalokaryocyte

*Rieder cell.

†Cytoplasm opaque. Plasma cells with azurophil granules are very rare.

He found that in cultures of human blood, by a technic which, with marrow, permits multiplication, maturation, and functional activity of cells of the granulocyte series for at least six weeks, the duration of life of the neutrophiles averaged sixty-one hours and, in the majority of instances, was between forty-eight and ninety hours. It seemed probable that these figures represented about the actual duration of life of these cells in the blood. The duration of the life of eosinophiles, from eight to twelve days, and basophiles, from twelve to fifteen days, determined by this method, is much longer than the duration of life of the neutrophiles.

In Table 79 from Osgood's article are collected the facts regarding the cells with azurophil granules. Table 80 from Osgood's article shows the facts relating to the cells with no granules.

¹Loc. cit. page 945.

Table 80 from Osgood's article shows the facts relating to the cells with no granules.

TABLE 80.—NO GRANULES (ALL HAVE ROUND OR OVAL NUCLEI)

CYTOPLASM	NUCLEOLI	CHROMATIN STRUCTURE	DIAMETER OF NUCLEUS IN RELATION TO DIAMETER OF CELL	SIZE OF CELL IN RELATION TO NEUTROPHIL LOBOCYTE	PEROXIDASE STAIN	NAME OF CELL
Opaque	Absent	Pycnotic	Less than half	Smaller	Negative	Metakaryocyte*† (Normoblast)
		Coarse	Less than two-thirds	Smaller	Negative	Karyocyte*† (Pronormoblast)
			More than two-thirds	Same or larger	Negative	Prokaryocyte* (Erythroblast)
		Coarse	Less than half	Usually larger	Negative	Plasmacyte†
	Present	Fine	Less than half	Usually larger	Negative	Proplasmacyte
			Less than two-thirds	Usually larger	Negative	Plasmablast
	Present	Fine	More than two-thirds	Same or larger	Negative	Karyoblast* (Megaloblast)
			More than half	Usually larger	Negative	Lymphoblast, monoblast, granuloblast (myeloblast)
Trans- parent	Present or absent	Coarse	More than half	Larger	Positive	Progranulocyte A (Promyelo- cyte II)
				Same or smaller	Negative	Prolymphocyte
					Negative	Lymphocyte

*May or may not contain hemoglobin. Other nucleated cells never contain hemoglobin.

†Sometimes two or more nuclei in one cell.

SPLENIC PUNCTURE¹

Splenic puncture requires withdrawal of splenic pulp without blood. Use oxidation-proof needle of steel or nickel, diameter 8 to 10 mm., and an absolutely air-tight syringe.

Produce superficial anesthesia by means of an intracutaneous Novocain wheal. Insert the needle into the superficial muscular plane, strictly in the zone of splenic dullness, immobilize the patient by asking him to hold his breath, give a short, but decided thrust, and rapidly and energetically aspirate, permitting the piston to return into its position of rest before the needle is withdrawn. Patient remains in bed for 12 to 24 hours. Ice is constantly applied to splenic region to prevent internal hemorrhage.

Splenograms are made by staining according to Wright-Giemsa (page 941), studying the aspect of the red cells and the relative number of hemato- blasts. The normal percentages are:

Neutrophilic polynuclears	20 to 30%
Eosinophilic polynuclears	1%
Basophilic polynuclears	Rare
Typical lymphocytes	50 to 60%
Medium mononuclears	10 to 20%
Monocytes	5 to 10%
Plasmacytes	1 to 2%
Normoblasts	20%
Naked nuclei	Numerous
Background, thick, sometimes granular	
Erythrocytes, normal	
Platelets, very numerous	

¹Perles, Suzanne: Paris méd. 25: 217-223, 1935.

The splenogram shows closer and more numerous cells than the corresponding hemogram. Abnormal erythrocytes are lacking. Platelets are present in larger numbers than in the peripheral blood. Determination of their number and coloration is one of the chief elements of splenic puncture.

Typical splenograms for each of the various pathologic reactions of the spleen are:

Lymphomatosis.—The splenogram of the lymphocytic leukemias is remarkable for homogeneity, achieved by the abundance of white elements, expressing the hyperplasia. Adult and young lymphocytes stand out from a clear and also homogeneous background. The young lymphocytes are much more numerous than in a normal splenogram (30 to 50, instead of 0.5 to 3 per cent), although the presence of all intermediary ages between a lymphoblast and a lymphocyte makes it difficult to make a clean differentiation. Polynuclear monocytes and plasmaocytes and especially normoblasts and megakaryoblasts are scarce, the latter even exceptional.

The uniformity and constancy of the splenic puncture observed in the lymphomatous diseases are a powerful argument for uniting forms apparently so far from each other as lymphocytic leukemias and pure splenic lymphomas.

Myelomatosis.—The splenogram of the myelomatoses is marked by a variegated aspect, which is due to the great changes of the elements of the myeloid series under the influence of age, and also to the more or less marked, but always noticeable, prevalence of a normomegakaryoblastic, exceptionally, megakaryocytic reaction. This notwithstanding, the myelomatous splenogram is a distinct and isolated type. The characteristic cells abound: myelocytes of all ages and all sizes, metamyelocytes, promyelocytes, primitive cells, hemohistioblasts, hemocytoblasts, polynuclears, and erythroblasts. The background is thick and granular, containing numerous platelets and poorly colored red globules.

Leukoblastoses.—The splenogram of acute leukemia shows a marked prevalence of the typical primitive cells (90 to 95 per cent). The other cells are more mature, yet decidedly young. These elements permit the tracing of the origin of the affection: lymphomatosis, myelomatosis, or erythroblastomatosis.

Cryptoleukemias.—A new chapter on leukemias, namely, one on atypical leukemias and cryptoleukemias (aleukemias or subleukemia) was written in terms of splenic puncture. These conditions were not capable of diagnosis by the ordinary methods of investigation. Whether or not the peripheral blood shows abnormal elements, the splenogram shows pullulation in the center of the atypical young cells.

SPLENIC TUMOR AND HEMATOLOGIC DIAGNOSIS

Enlargements of the spleen are very common. It is important to classify them, if possible, from clinical and hematologic standpoints. In blood diseases, the histologic structure of the splenic tumor is largely dependent upon which blood system is affected; for instance, the pulp, in cases of myelocytic leukemia, shows an increase in the myeloid elements; in lymphocytic leukemia, the lymphatic elements of the pulp increase; and in monocytic leukemia, there are many monocytes in the pulp. In polycythemia there is a marked hyper-

emia of the spleen and often a myeloid metaplasia. There is a disturbance in the spleen in purpura werlhofii, also in chronic leukemia, in aleukemia, and panmyelophthisis. It is interesting to note that benefit results in purpura werlhofii as well as in some cases of leukemia following splenectomy. In malignant tumors of the spleen, lymphocytes are found in the pulp, as in carcinoma, lymphatic sarcoma, etc. Single metastases in the spleen are rare. In benign megalo-splenia, due to reticular system disturbances, such as Gaucher's disease, Niemann's disease, etc., the spleen is filled with these substances such as cerasus phosphatin or cholesterolin. This can be determined by splenic puncture. The blood picture in these cases is not characteristic. In certain cases, there are anemia, leukopenia, and thrombocytopenia. There is a special form of splenic tumor seen in osteosclerosis. In these cases there is a chronic constitutional anemia.

In constitutional anemias, there are splenic disturbances by trauma, cirrhosis, postinfectious scars, etc. Banti's disease shows splenic tumor with anemia, later splenic and liver cirrhosis with ascites and cachexia.

In certain splenic tumor diseases, one notes increased urobilin and urobilinogen in the urine and chronic subicterus. Splenic tumors are due either to the breaking down of the poorly resistant erythrocytes as in icterus hemolyticus, or to deposited erythrocytes as in pernicious anemia or blood poisoning.

If the splenic function is markedly disturbed, one sees polychromasia, eosinophilia, with pathologic erythrocytic forms, an appearance of normoblasts, erythrocytes, inner bodies, etc. It is probable that the splenic organ in these cases will show a sclerosis, also at times atrophy if splenic decrease in size has been seen.

Infections produce splenic tumors; for instance, protozoal diseases, malaria, kala-azar, etc. Then there are the acute inflammatory tumors of the spleen in bacterial infections, especially typhoid fever, brucellosis, and sepsis. There is a chronic inflammatory enlargement of the spleen in granulomatosis, such as syphilis, tuberculosis, lymphogranuloma. The diagnosis may often be clarified by puncture of the spleen; for instance, one may find the characteristic cells of lymphogranuloma with eosinophiles in the splenic puncture. Upon puncture one may find malaria pigment.

Summary of the Causes of Splenic Enlargement

They may be grouped under the following headings: (1) mechanical obstruction; in other words, venous congestion; (2) infective and toxic conditions; (3) blood diseases; and (4) reticulososes and reticuloendothelioses—with or without disturbance of lipoid metabolism; (5) hemorrhages.

1. Venous or Passive Congestion of the Spleen.—

This may occur rapidly from failure of the right heart or from thrombosis or thrombophlebitis of splenic or portal veins. It may occur slowly from some central cause such as mitral stenosis or incompetence either alone or associated with changes at the tricuspid orifice, adherent mediastinopericarditis, chronic fibroid myocarditis, emphysema of the lung with chronic bronchitis, interstitial pneumonia, or adherent pleurisy.

Additional *local* causes are (1) cirrhosis of the liver; (2) tumors of the liver; (3) chronic inflammatory thickening along the portal tracts, either

syphilitic or tuberculous; (4) pressure upon splenic vein by slowly growing carcinoma of the head of the pancreas; (5) by enlarged lymph glands in the hilum of the liver; (6) aneurysm.

2. Enlargement Due to Acute and Chronic Infective and Toxic Conditions.

—Acute infective and toxic conditions produce enlarged spleen—hyperemic, proliferative, and infiltrative. This acute splenitis on cut section shows the pulp coming off on the knife, the “diffuent spleen.”

Chronic infective enlargements are due to tropical diseases, malaria, and kala-azar, or syphilis and tuberculosis, including in this cirrhosis of the liver.

This initial hyperemia is replaced by hyperplasia of the splenic tissue, especially of the pulp, so that the color is frequently paler than normal, the consistency either normal or a little increased, the capsule thickened. Later, hyperplasia of the stroma occurs with thickening of the organ. The sheaths of the vessels and sometimes the trabeculae show hyperplasia of connective tissue. This may cause atrophy of the cellular constituents in the pulp. Cut sections show distinct mottling with lighter and darker red parts. At times there is marked pigmentation, and frequently there is adherent perisplenitis.

Spleen in Malaria.—There are found acute inflammatory swelling of the spleen and melanin and hematogenous pigment contained in free or fixed phagocytes, or lying free in the sinuses. Parasites are found in the red blood cells of the pulp and in the large phagocytes. In chronic malaria, the spleen is harder from increase of the reticulum, which predominates over the cellular hyperplasia—color is gray brown.

Spleen in Kala-Azar.—Enlargement is associated with marked anemia, leukopenia, skin hemorrhages, local dropsies, and enlargement of the liver, and exceeds enlargement due to malaria. Microscopically, many large mononuclear histiocytic phagocytes, 4 to 5 times the diameter of a red blood corpuscle, are seen containing numerous oval parasites. Other cells of smaller size lying in the pulp contain red blood cells and pigment. In later stages there is considerable increase of the reticulum, especially near the malpighian bodies.

Tuberculosis of the Spleen.—This is secondary, or there may be tubercles from acute miliary tuberculosis or subacute and chronic tubercles with caseation. In children a bovine origin of the infection is suggested.

In acquired *syphilis* the spleen is enlarged, especially in the tertiary stage, with waxy or amyloid infiltration. In congenital syphilis in children enlargement associated with recent or old perisplenitis, accompanied by diffuse fibrosis, endarteritis, and occasionally periarteritis, numerous spirochetes can be demonstrated.

Increase of reticulum is seen in leprosy, with enlargement, perisplenitis, atrophy of malpighian bodies, and disappearance of lymphocytes. There are numerous leprosy cells in clusters.

3. **Splenic Enlargements in Blood Diseases.**—Leukocythemias, splenic anemia and Banti's disease, and polycythemia.

4. **Splenomegalies in the Reticuloses and Reticuloendothelioses.**—This includes active hyperplasia of the true reticulum, with changes in follicles of lymph glands and in malpighian bodies of the spleen. In reticuloses, the spleen

may weigh as much as 4.7 kilograms. Red pulp is described with large malpighian bodies, the ordinary lymphocyte being crowded out. Large cells are seen.

The Spleen in Lymphadenoma.—Enlargement is uniform, capsule thickened. Section shows congestion, brownish-red color; malpighian bodies are prominent. There is an accompanying alteration of the lymph glands of the body. Lymphadenoma cells with nongranular cytoplasm and convoluted, indented, or ring-shaped nuclei, closely resembling the giant cells of bone marrow, are present. Eosinophiles are present.

Reticuloses Associated With Disturbance of Lipoid Metabolism.—

a. Gaucher's disease: Spleen may weigh from 3,000 to 4,000 gm. Pulp is infiltrated with Gaucher cells.

b. Niemann-Pick's disease is found in children, especially females, but, in contrast with Gaucher's disease, its course is rapid and leads to death within three years. Lipoids are found in the spleen. Scar formation in the later stages replaces the lipoid formations. These changes must be distinguished from those occurring in amaurotic familial idiocy, known as Tay-Sachs' disease, which may occur simultaneously. The visceral changes in Niemann-Pick's disease do not occur in Tay-Sachs' disease, nor do all cases of Niemann-Pick's disease show the cerebral changes of Tay-Sachs' disease, in which the brain is hard and leathery, the fissures are widened, the corpus callosum atrophied, and fat granule cells collect about the blood vessels.

c. Xanthogranulomatosis is also known as the Hand-Schüller-Christian disease. This disease attacks males and is often associated with exophthalmos and diabetes insipidus. Deposits of cholesterol esters within large phagocytic cells fill multiple defects in the skull; there are also deposits in the white matter of the brain and cord, in periosteum of other bones, in lungs, pleura, liver, lymph glands, bone marrow, and spleen.

5. **Splenomegaly in Presence of Hemorrhages.**—This enlargement and the edematous softening of the spleen occur whenever there have been blood effusions and hemorrhages of any considerable size in any organ or cavity of the body. It is due to increased function, or blood destruction, in the main.

Tumors of the Spleen.—Primary fibroma, chondroma, osteoma, lymphangioma, hemangioma, and melanoma of the spleen have been recorded. Of secondary tumors, sarcomas occur less infrequently than carcinoma. The most common type is melanotic sarcoma.

Animal Parasites.—Most frequent are the hydatids of *Echinococcus granulosus*.

Cysts of the spleen are rare. True cysts come from dilatation of blood spaces, from cavernous angioma, or dermoids; or they may be parasitic. False cysts may be traumatic, due to necrosis with softening, or tuberculous.

Abscess of the spleen is seen from breaking down, as in sepsis.

Gaucher's Disease

In the consideration of Gaucher's disease, one must bear in mind that it belongs to the group of diseases known as xanthomatosis, or a series of clinical

and anatomic conditions in which lipoid substances are deposited in certain tissue systems or organs of the body. Xanthomatoses have been classified as follows: (1) generalized; (2) local.

There are two generalized types, the symptomatic and the essential or primary.

The symptomatic form is seen as multiple xanthomata in diabetes or liver diseases which are associated with jaundice, also in chronic kidney disease. These xanthomata occur as yellow pea-like nodules in the skin. In such cases, lipoid is deposited in proliferating cells of the connective tissue of the skin and in the endothelium of lymph vessels. These large cells contain fat.

The second type of the generalized form, the essential or primary xanthomatoses, comprises Gaucher's disease, Niemann-Pick's disease, Hand-Schüller-Christian's disease, and fourth, the primary nonsymptomatic external and internal xanthomatoses in skin and the inner organs.

Gaucher's disease was first described by Gaucher in 1882. He called it a primary epithelioma of the spleen. In 1900, Bovaird of New York called it a nonneoplastic disease, stating that it was an endothelial hyperplasia in the spleen, liver, and lymph nodes. In 1907, Schlagenhauser called it a systematic affection of the reticulum cells of all lymphatic and hematopoietic organs, including the bone marrow. In 1907, Marchand recognized the importance of the deposit of foreign substances in the histogenesis of the Gaucher cells. In 1916, Mandelbaum and Downey found conception of the origin of these cells. In 1924, Lieb and Epstein, by study of the spleen, showed the lipoidal nature of the Gaucher substance, showing that kersasin is its important constituent. Kersasin belongs to the cerebrosides (sphingogalactides). This disease is not rare. Pick in 1926 collected thirty-nine cases to that date. Cushing and Stout, in the same year, collected in this country fifty cases. Hoffman and Makler¹ collected eighty-nine cases in all, including their own case.

The disease is of familial incidence. Collier first reported its occurrence in more than one member of a family. It is more common in the female.

It is characterized by splenic and liver tumors. The oldest patient of L. Pick was fifty-six years. The bone was involved as noted by pain in the lower ends of the femur and tibia or tenderness over sternum and ribs. The earliest signs are the characteristic pigmentation of the skin and definite blood changes. The skin pigmentation, mostly confined to face, neck, and hands, is yellow brown to ochre. There also occur brownish-yellow wedge-shaped thickenings of the sclerae, the base of the wedge lying toward the margin of the cornea. These pigmentations are expressions of a general hemochromatosis which is constantly present in this disease and becomes more marked with increased duration of its course.

Hematologic Findings. —

The *blood changes* are leukopenia, which may be due to diminution of neutrophiles or lymphocytes, a moderate hypochromic anemia, often a thrombocytopenia, often bleeding from the nose or gums, hematemesis, melena, or uterine bleeding. Even slightest trauma produces a purpuric bleeding. Death

¹Hoffman, S. J., and Makler, M. I.: *Am. J. Dis. Child.* 38: 775, 1929.

is not usually due to cachexia, but more often to an intercurrent disease, such as pneumonia, sepsis, tuberculosis, or carcinoma. Some of these patients die following splenectomy. Bone marrow puncture shows Gaucher type of cell.

Pathologic Anatomy: A very large spleen, weighing as much as 8,100 grams. Cut section shows a reddish-gray, pink, or sometimes chocolate-brown background stippled with innumerable gray-white or gray-yellow markings, corresponding to accumulations of Gaucher cells. Second, small or large nodular cavernous foci form in spleen and are always multiple. Liver may weigh 3,300 gm. Remember the normal weight is 1,500 gm.

The lymph nodes show no enlargement of the superficial glands, but enlargement and pigmentation of the intrathoracic and intraabdominal glands occur. Hemachromatosis of skin, sclerae, parenchyma of spleen, liver, and lymph nodes are also present.

The essential histologic change is the presence of many Gaucher cells which are demonstrable by acid fuchsin stain after picric acid fixation. Cytoplasm is wrinkled giving them a vacuolated appearance. There are arterial branches within the nests of Gaucher cells. Pigment is absent only in children and infants. Owing to the disappearance of Gaucher cells, we see fibrotic nodules. In the liver, the process is cirrhotic and contains nests of Gaucher cells.

The peculiar appearance of the Gaucher cells is due to the presence of kersin, which may be demonstrated chemically even in dried spleens. The formula of kersin given by Rosenheim is $C_{47}H_{91}NO_8H_2O$. It has properties similar to phrenosin, and accompanies phrenosin in tissues of the nervous system, both constituting a cerebroside or glycolipin. Capper, Epstein, and Schless¹ report a case of Gaucher's disease with presentation of a table differentiating the lipid disturbances. This is Table 81 on pages 954 and 955.

Method for demonstration of kersin is given by Bloom and Kern² and is carried out by taking formaldehyde-fixed spleen, or fresh spleen, grinding through a meat chopper, drying to a constant weight in a vacuum desiccator. The dried powder is then extracted for 5 days with ether in a Soxhlet. The residue is extracted with ethyl alcohol at 40° C. for 36 hours. The material is further extracted at boiling temperature in a fresh portion of ethyl alcohol for 5 days. The ether soluble fraction is treated with acetone, the resulting precipitate of phosphatids filtered off, washed with acetone, and dried to constant weight in the vacuum desiccator. The acetone and ether are removed from the filtrate, and the residue, containing cholesterol and fatty acids, dried to constant weight.

That part of the precipitate from the 40° C. alcohol fraction is dissolved in 100 c.c. of alcohol, to which are added 30 c.c. of cold saturated mercuric chloride. This gives a colorless gelatinous precipitate, much like egg white in appearance, which fills the entire solution. The precipitate is removed by filtration and suspended in methyl alcohol; hydrogen sulphide is passed through the suspension. The precipitate is filtered off, the filtrate boiled with charcoal and filtered again. This filtrate when concentrated and cooled gives an almost white, gelatinous precipitate. Under the microscope this appears as round crystals arranged radially about a nucleus. On hydrolysis of the material for 8 hours in methyl alcohol and sulphuric acid under a reflux condenser, a white, crystalline material with a melting point of 56.7° C. is obtained, which corresponds very closely to that of the methyl ester of lignoceric acid.

¹Capper, A., Epstein, H., and Schless, R. A.: *Am. J. M. Sc.* 188: 84, 1934.

²Bloom, Wm., and Kern, Ruth: *Arch. Int. Med.* 39: 456, 1927.

TABLE 81.—XANTHOMATOSSES OR LIPOIDOSES—DISEASES DUE TO DISTURBANCES IN LIPOID METABOLISM*
(Rowland.) (Pick.)

NAME	TYPE OF LIPOID	BLOOD FINDINGS	EYE-GROUNDS	SPLEEN, LIVER GLANDS	AGE	BONES	PATHOGNOMONIC FEATURES	SKIN	TREATMENT	REMARKS
A. Gaucher's disease (90-100 cases)	Cerebrosid (kerasino-sphingogalactid)	1. Hypochromic anemia 2. Leukopenia 3. Thrombopenia 4. Later, hemorrhagic diathesis	Negative	Spleen markedly enlarged Liver enlarged Slight or no lymphadenopathy	Infancy to adult life	Mottling and rarefaction with cortical thinning, flasklike appearance ends of femora	1. X-ray appearance of long bones, especially femora 2. Splenic puncture 3. Bone-marrow examination	Subicteric pigmentation of exposed parts in 45%. Yellow thickened pingueculae in eyes in 15%	Splenectomy (fatal in 20%) or radiation (both palliative)	Common in Jews
B. Niemann-Pick's disease or lipoid histiocytosis (Bloom)	Phosphatids	1. Moderate secondary anemia 2. Slight leukocytosis 3. Vacuolization of non-granulated cells 4. Excess of lipoids in blood	Cherry-red spot in macula occasionally found	Spleen and liver very large Moderate lymphadenopathy	Infancy	Any bones or organs may be invaded by the foam cells	Splenic puncture	Brownish-yellow discoloration	No treatment, all fatal	Mental retardation may be present resembling Tay-Sachs' disease. Most common in Jews

C. Hand-Schüller-Christian disease (45-50 cases)	Cholesterol and its esters	There may be a hypercholesterolemia	Negative	Not enlarged	Any age	Membranous bones involved	1. Defects in membranous bones 2. Diabetes insipidus 3. Exophthalmos 4. Gingivitis and stomatitis 5. Adiposogenital dystrophy	Negative	X-ray treatment of involved area causes prompt local healing but does not stop the progress of the disease	Brain shows prelipoid deposits in granular layer and about dendrites of the Purkinje cells. Most common in Jews
D. Tay-Sachs' disease or amaurotic familial idiocy	A pre-lipoid	Negative	Cherry-red spot in macula lutea usually found	Usually negative	Infancy	Not involved	1. Eyegrounds 2. Mental retardation 3. Hyperacusis	Negative	All fatal	
E. Xanthomatoses of skin, etc.	Cholesterol and its esters	Negative	Negative	Negative	Any age	Any bones may be involved	-----	Xanthomatoses of any part of skin	Excision, if tumorous	

*Capper, Epstein and Schless: Am. J. M. Sc. 188: 84, 1934.

It is interesting to note that Gaucher cells arise from the endothelium of blood vessels. It is evidently a primary disease of the reticuloendothelial system, based upon an error in metabolism. The pigmentation is due to the fact that lipoid-storing cells of every sort show a tendency to store simultaneously hemoglobinogenic pigments; with stimulation of the reticuloendothelial structures, there is an increased hemosiderosis, that is, an increased blood destruction. The mild anemia, which is constant in this disease, is due to the bone marrow penetration by the Gaucher cells.

The bony changes are demonstrable by roentgenologic examination.

The **blood picture** shows a low hemoglobin and a low red blood cell volume. The following hemogram is illustrative of Gaucher's disease:

Hemoglobin, 44 per cent; red cells, 3,628,000; white cells, 2,650. Neutrophils 49 per cent; lymphocytes 38 per cent; eosinophiles 5 per cent; monocytes 8 per cent; basophiles 0; blood platelets 30,000.

The anemia in this disease is not as well marked as it is in Banti's disease.

Niemann-Pick Disease

This disease is similar to Gaucher's disease from a histologic standpoint. It is a typical disturbance of the histiocyte apparatus. There is a great enlargement of the liver, a change which occurs much more rapidly than it does in Gaucher's disease.

The blood picture is similar to that of Gaucher's disease.

Anatomically, Niemann-Pick disease differs from Gaucher's disease in that the characteristic cells are much more prevalent in the spleen, liver, lymph glands, and bone marrow. Reuben maintains that the anemia of Niemann-Pick disease is not as severe as that of Gaucher's disease, nor is the leukopenia as pronounced. He also states that the qualitative leukocytic blood picture is unchanged. Moschokowitz reported a case with 33 per cent hemoglobin, 1.6 million red cells, and 6,500 white cells, of which 90 per cent were neutrophils. According to Reuben the prognosis in children is more severe than in adults, and it is a more rapidly fatal disease. It seems to be associated with delayed development of the blood.

RELATIONSHIP OF THE LIVER TO BLOOD DISORDERS AND DISEASES OF THE RETICULOENDOTHELIAL SYSTEM¹

The liver participates in blood disorders by virtue of its function as a reticuloendothelial organ, its role in the metabolism of blood pigments, in the storage of iron, in the synthesis of blood proteins, and in the storage of the anti-pernicious anemia factor. Furthermore, the spleen, which participates in so many blood dyscrasias, bears an intimate relationship to the liver both physiologically and pathologically.

In most blood dyscrasias, the liver is affected to some extent, and it may be markedly enlarged. However, liver function is seldom significantly impaired. Green and Conner in 1926 and other investigators since then have shown that all of the commonly used liver function tests usually give normal results in cases of hepatomegaly due to leukemia, polycythemia, hemolytic jaundice.

¹Reinhard, Edward H.; Bull. St. Louis M. Soc., May 11, 1945; Lab. Digest 9: 3, 1945.

pernicious anemia, and even Gaucher's disease. In polycythemia the liver is frequently enlarged due to chronic hyperemia, in leukemia there may be marked infiltration of the liver with leukemic cells, in pernicious anemia the hepatomegaly which is sometimes seen is due to fatty changes and hemosiderin deposits, while in Gaucher's disease, Hodgkin's disease, lymphosarcoma, and several other diseases of the reticuloendothelial system striking pathologic changes may be found in the liver. The fact that these diseases rarely result in any significant impairment of liver function either clinically or by laboratory testing is a manifestation of the great reserve power of the liver.

Jaundice is common in the blood dyscrasias, and this sometimes leads to an incorrect diagnosis of primary hepatic or biliary tract disease. In congenital or acquired hemolytic icterus, the jaundice is obviously due to the rapid breakdown of red blood cells as a result of either an inherent fragility of the erythrocytes or possibly due to the action of a hemolysin. In pernicious anemia the characteristic lemon yellow color is due either to an abnormality of pigment metabolism or to hemolysis.

Rich and associates have suggested that in any hemolytic anemia the jaundice may be due to a combination of rapid disintegration of erythrocytes with hepatic lesion. It is very difficult to produce jaundice in a dog by the injection of bilirubin. Furthermore, Rich showed that simple hemorrhage in dogs as well as pernicious anemia and secondary anemias in human beings may be accompanied by damage to the cells around the efferent veins of the liver lobules. This lesion is identical with that seen in chronic passive congestion of the liver, and Rich postulated that the lesion is due to tissue anoxia resulting from the anemia. The jaundice is thus due to a combination of hemolysis plus functional liver damage. In human hemolytic anemias, a hepatic lesion is not always demonstrable histologically, but it is probably true that a perfectly normal liver can excrete all the bilirubin the body is capable of producing; hemolysis alone, therefore, probably cannot produce jaundice unless the liver is functionally deranged.

In leukemia, as well as in the so-called lymphomas, mild jaundice sometimes occurs due to infiltration of the liver or destruction of liver cells by the lymphomatous process. Occasionally enlarged lymph nodes in the portal fissure may occlude the bile ducts and produce marked jaundice. Jaundice due to this mechanism may be completely relieved by x-ray therapy directed to the upper abdomen.

Hemorrhagic Disorders Due to Diseases of the Liver or Biliary Tract.—In any hemorrhagic disease there is always either a defect of capillary permeability or a defect in the coagulation mechanism. So far as is known, the liver has nothing to do with capillary permeability, but the liver does contribute to blood coagulation. There are four essential substances which must be present in order for blood to coagulate normally; these are thromboplastin, Ca ions, prothrombin, and fibrinogen. Of these, fibrinogen is probably synthesized solely in the liver, whereas prothrombin is synthesized primarily in the liver. Thus, theoretically, liver disease may result in hemorrhage due to failure of synthesis of either of these factors.

Actually, a condition known as congenital fibrinopenia has been described in which there is a congenital specific defect in the ability of the liver to synthesize fibrinogen; however, only half a dozen cases have been described. The amount of fibrinogen normally present in the plasma is far in excess of what is required for coagulation. In any kind of severe liver disease, the plasma fibrinogen level may be decreased below normal, but this is always overshadowed by the much more marked and more significant decrease in prothrombin.

One of the factors that may produce bleeding due to inadequate amounts of prothrombin in the blood is vitamin K deficiency. Dietary deficiency never produces vitamin K deficiency in adult human beings, as proteolytic bacteria in the intestines can synthesize all the vitamin K that is required. However, during the first few days of life there are few if any bacteria in the intestines and in the newborn infant prothrombin deficiency is common. This is generally accepted as being the principal factor responsible for hemorrhagic disease of the newborn.

In adults, vitamin K deficiency occurs only when there is poor absorption from the intestinal tract due either to severe diarrhea or to absence of bile salts in the gut. Vitamin K is fat soluble and is not absorbed in the absence of bile salts. Thus, the bleeding which occurs in severe obstructive jaundice can be corrected either by the parenteral administration of vitamin K or by the administration of bile salts by mouth.

In contrast with this type of prothrombin deficiency, the prothrombin deficiency resulting from severe liver damage is not affected by vitamin K administration. When the liver cells which synthesize prothrombin are destroyed, the only rational therapeutic approach is to keep the patient alive by supplying prothrombin from without in the form of plasma transfusions. This only stops the bleeding temporarily, but is an extremely important procedure whenever the nature of the liver disease is such that regeneration of liver tissue is possible.

A condition known as congenital hypoprothrombinemia has been observed in a few children in which the liver lacks the ability to synthesize prothrombin. There have been very few cases of this sort reported in the literature, but now that it is being looked for, it is being recognized more frequently. Quick stated that he has knowledge of over half a dozen such cases himself.

The Role Played by the Liver in Certain Anemias.—The liver plays an important part in two types of anemia—pernicious anemia and iron deficiency anemia. It is well established that the erythrocyte maturation factor is stored in the liver, and the liver of patients with pernicious anemia lacks this substance. It has been shown that cirrhotic livers are deficient in erythrocyte maturation factor. It is, therefore, very easy to assume that the anemia so commonly seen in various types of liver disease is merely due to the absence or diminished quantity of this factor in the liver. It is known that some degree of anemia is usually present in advanced cirrhosis of the liver. But perhaps sufficient emphasis has not been placed on the fact that in only about half of these cases is the anemia macrocytic. The mean corpuscular volume in general is in the vicinity of 100 cubic microns; however, the macrocytosis does seem to increase as the anemia becomes more marked. The anemia of cirrhosis is often accompanied by a reticulocytosis and occasionally by mild leukopenia.

It has frequently been suggested that because of the close resemblance of the blood picture to that of pernicious anemia, the damaged liver is responsible for a deficiency of antipernicious anemia factor. A reticulocyte response to the administration of liver extract does occur in some of these cases, but in other cases of cirrhosis no such effect is obtained. It is probable that the anemia of cirrhosis and other types of severe liver disease is not due to deficiency of the erythrocyte maturation factor. It is important to remember that macrocytosis which is so characteristic of pernicious anemia also occurs in the anemias of chronic uremia and occasionally in leukemia. Furthermore, in normal animals the kidney as well as the liver contains large amounts of anti-pernicious anemia factor and, thus, it is hard to see how liver disease alone could produce a significant decrease in the amount of available erythrocyte maturation factor unless the kidneys were simultaneously damaged.

Castle studied the macrocytic anemias due to nutritional deficiency. In two of the three patients he studied, the anemia developed during pregnancy. In spite of the fact that the anemias in all three of these patients were strikingly macrocytic, no reticulocyte response occurred in any of them following the intramuscular injection of liver extract. In all three cases, however, a reticulocyte response followed by gradual rise in red blood counts occurred when liver extract was given by mouth. Castle concluded that the "unitarian" hypothesis which regards all macrocytic anemias as being due to deficiency of the erythrocyte maturation factor is not tenable. He suggested that in this type of nutritional macrocytic anemia the digestive organs form a new hematopoietic substance from the liver extract in a fashion analogous to the food-gastric factor relationship in true Addisonian pernicious anemia. Present evidence does not justify the conclusion that all macrocytic anemias are due to the same mechanism; there is no conclusive proof that the macrocytic anemia of liver disease is due to deficiency of the anti-pernicious anemia factor.

When red blood cells are hemolyzed in the body, the hemoglobin is broken down and the iron thus liberated is stored in the reticuloendothelial tissues throughout the body. The liver is perhaps the most important of all the organs which store iron. By virtue of this storage mechanism, extremely minute quantities of iron are lost from the body by excretion. It is for this reason that hypochromic microcytic anemia due to iron deficiency practically never occurs except in hemorrhagic states where large amounts of actual hemoglobin are lost from the body. Hypochromic microcytic anemia due to iron deficiency very rarely, if ever, occurs as a result of inadequate intake of iron in the diet. Thus, the liver plays an important part in iron metabolism.

THE SEDIMENTATION RATE OF ERYTHROCYTES*

John Hunter in 1797 is given the credit of first noting that the speed of sedimentation of the erythrocytes was increased in individuals suffering from inflammatory lesions and that this speed varied with the severity of the infection. Others (Nasse, 1836; Davy, 1839; Muller, 1844) noted the behavior

*Part of this section is from a monograph by R. B. H. Gradwohl, "The Rediscovery of Blood Sedimentation Test," *Rev. de med. Trop. y Parasit. Bacteriol.* 8: 295, 1937.

of red cells but did not explain it. Interest was renewed by Fahraeus¹ in 1917. In 1920 Linzenmeier² worked out a technic. In this country, Friedlander,³ Polak and Mazzola,⁴ Baer and Reis⁵ reported the value of the test in obstetrics and gynecology. Gruenfeld,⁶ Cutler,⁷ Bach and Hill,⁸ Bannick,⁹ and others have called attention to its usefulness in medical cases, particularly rheumatic fever and tuberculosis. Grodinsky, Lesser and Goldberger, and Smith have written of its advantages in general surgery.

Sedimentation of the erythrocytes is a very complex biological process, the basic mechanisms of which cannot be reduced to a common denominator. Fahraeus referred to the phenomenon as "suspension instability," which is still a good description. The physicochemical forces which have been implicated are numerous and varied. The electrical charge of erythrocytes as related to proteins has been thought to play a role. Hoeber, Mond, and Ley note that hydrogen ion concentration influences the rate, with acidosis slowing and alkalosis accelerating it. The plasma proteins exert an influence on the sedimentation rate. Fibrinogen is perhaps the most readily demonstrable protein affecting sedimentation. In diseases associated with hypofibrinogenemia, such as liver disease, the rate is very slow and some observers have suggested that the sedimentation test be included in liver profile studies. The addition of fibrinogen to normal blood in vitro markedly accelerates settling of the erythrocytes. A. J. Meyers and co-workers¹⁰ confirm this fact by in vitro experiments and call attention to alpha-2 globulin and gamma globulin as other protein fractions having a similar effect. These workers emphasize that the erythrocyte sedimentation rate alone "does not give any clue as to the level of any one of the responsible factors." They offer two complex formulas based on plasma:cell ratio and plasma protein concentrations from which formulas they can predict the sedimentation rate. The concentration of proteins, perhaps qualitatively as well as quantitatively, in some unknown manner affects the aggregation of erythrocytes in the form of rouleaux or agglomeration.

The specific gravity of plasma does not play an important role because the addition of weak saline causes a diminution in sedimentation rate.

A purely physical factor in sedimentation rates can be readily demonstrated and is now common knowledge. If one sedimentation tube is set up perfectly perpendicularly and another tube of the same specimen is aligned at an angle, the latter will have a higher speed of sedimentation than the former. The temperature under which the test is performed affects the rate, the rate tending to be elevated as the temperature rises. These two factors are important controls in performing the test.

¹Fahraeus: *Hygiea* 80: 369, 1918.

²Linzenmeier: *Ztschr. f. arztl. Fortbild.* 20: 445, 1923.

³Friedlander: *Am. J. Obst. & Gynec.* 7: 125, 1924.

⁴Polak and Mazzola: *Am. J. Obst. & Gynec.* 12: 700, 1926.

⁵Baer and Reis: *Am. J. Obst. & Gynec.* 12: 700, 1926.

⁶Gruenfeld: *J. M. Sc. New Jersey* 25: 577, 1928.

⁷Cutler, J. W.: *Am. J. M. Sc.* 183: 643, 1932.

⁸Bach and Hill: *Lancet* 1: 75, 1932.

⁹Bannick: *Proc. Staff Meet., Mayo Clin.* 8: 81, 1933.

¹⁰Meyers, A. J., Trevorrow, V., Washburn, A. H., and Mugrage, E. R.: *Blood* 8: 893, 1953.

Technic of Determining Sedimentation Rate of Erythrocytes

Materials.—

Westergren Method

Westergren pipette, 200 mm. in length, 2.5 mm. diameter, graduated in mm.

Westergren block.

Tourniquet.

Sterile 2 c.c. syringe.

Sterile 3.7% solution of sodium citrate.

Alcohol, sterile sponges, adhesive tape.

Watch glass.

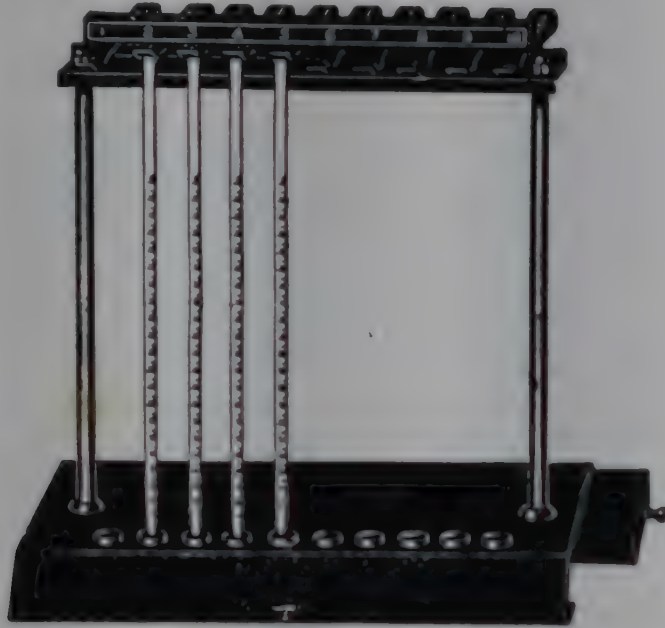


Fig. 224.—Westergren sedimentation apparatus, improved Hellige model. (Hellige, Long Island City, N. Y.)

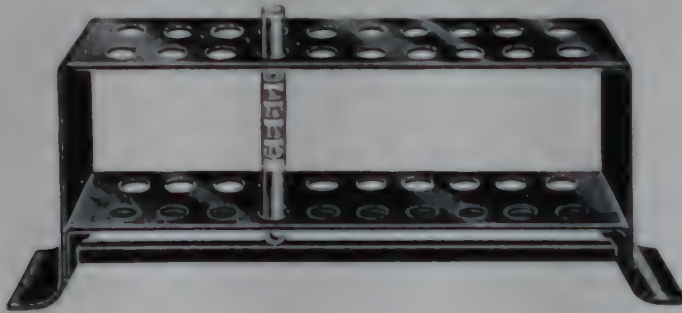


Fig. 225.—Rack, Linzenmeier or Cutler sedimentation tubes. (Hellige, Long Island City, N. Y.)

Technic.—

Draw sodium citrate solution into the syringe to the mark 0.4.

Apply the tourniquet to the patient's arm, and locate the vein.

After disinfecting the skin, insert the needle into the vein and draw blood into the syringe up to the mark 2 c.c. Remove tourniquet and withdraw the needle.

Remove needle from syringe.

Draw back the plunger of the syringe, and tilt back and forth several times to insure a good mixture of the blood and citrate.

Eject contents of syringe into watch glass.

Draw the mixture into a Westergren pipette to the mark 0, and place the pipette in a Westergren block in a vertical position.

The number of millimeters that the erythrocytes drop in the pipette is the sedimentation rate.

Read the sedimentation of the erythrocytes in mm. at the end of 1 hour.

Normal.—After 1 hour, 1 to 15 mm. for men; 0 to 20 mm. for women.

Linzenmeier's Method**Materials.—**

Tubes 5 mm. in diameter, 65 mm. in height, with two marks, one at the level of 1 c.c., the other 18 mm. below this. Other equipment same as above.

Technic.—

Draw 0.2 c.c. of 3.7% sodium citrate solution and 0.8 c.c. blood into the syringe and mix.

Pour the mixture into the tube, up to the 1 c.c. mark.

Allow to stand in an upright position until the red blood corpuscles have settled to the mark 18 mm.

Note the time for this to take place. Record in minutes.

Normal.—Healthy men, from 350 to 600 minutes; healthy women, from 300 to 600 minutes. Menstruation 600 minutes. Anything under 200 minutes is pathologic.



Fig. 226.—The Linzenmeier, Cutler, and Wintrobe tubes.

Materials.—**The Graphic or Cutler Method**

Tubes 5 c.c. capacity, graduated in 0.1 c.c., each 0.1 c.c. 1 mm. in height.

Sterile 5 c.c. syringe.

Freshly prepared 3% sodium citrate solution, sterile.

Other equipment same as above.

Sedimentation report charts which may be purchased from Charles M. Berkemeyer, Sellersville, Pa., and from Clay-Adams Co., Inc., 44 East Twenty-Third Street, New York, the A. S. Aloe Co., St. Louis, and other supply houses.

Technic.—

Draw into the syringe 0.5 c.c. sodium citrate solution.

Draw blood to mark 5 c.c.

Mix as above.

Pour contents into sedimentation tube.

Stopper with a paraffin-coated cork.

The tubes are allowed to stand 10 hours, but must be observed every 5 minutes for one hour.

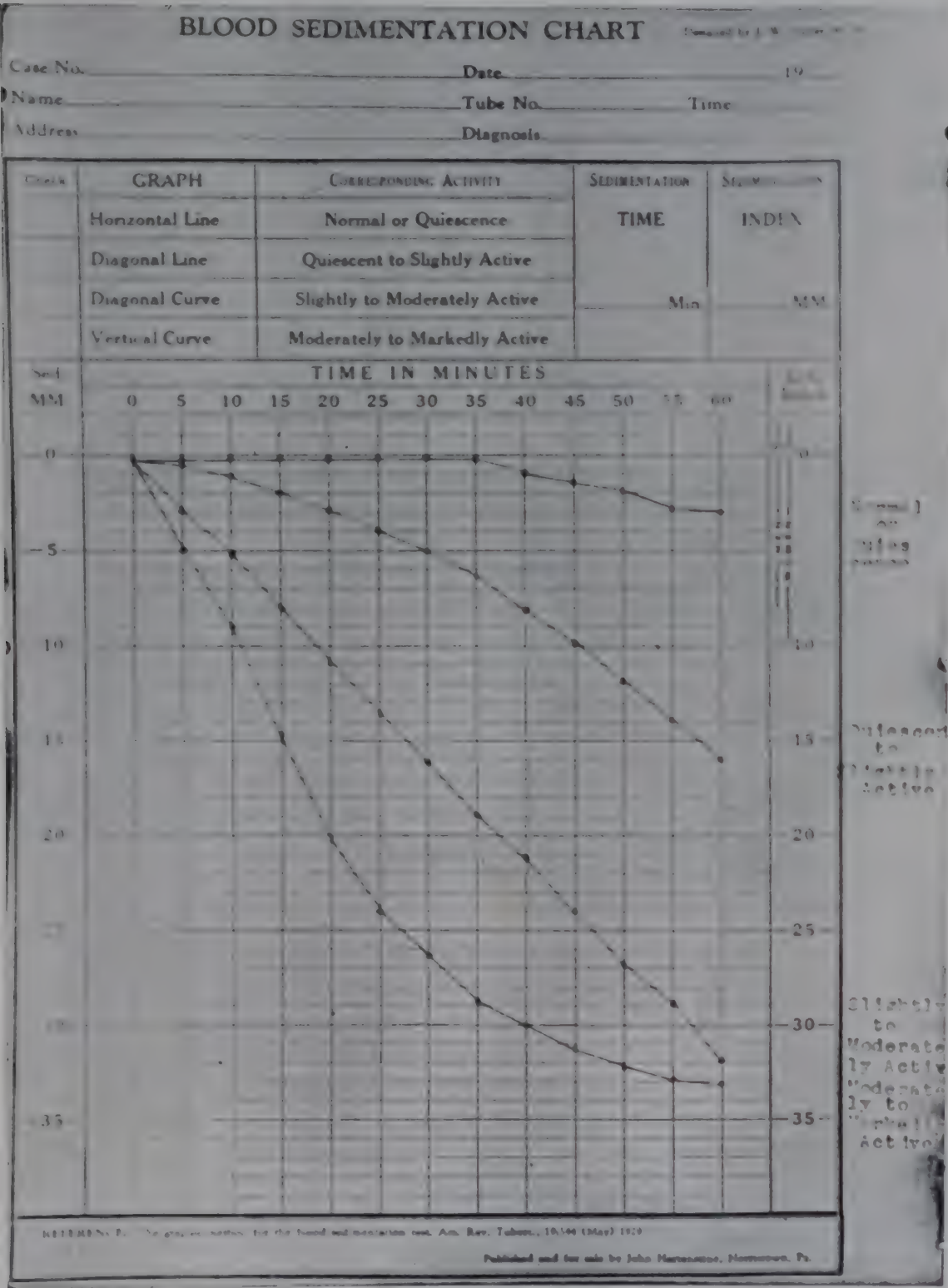


Fig. 227.—Cutler chart.

Draw curve on sedimentation chart.

The sedimentation value, determined by the sedimentation of the red cells in the first hour, depends upon the sedimentation graph, the sedimentation index, and the sedimentation time. There are four graphs, two straight lines, and two curves. They are called "horizontal" line, "diagonal" line, "diagonal" curve, and "vertical" curve.

Sedimentation index is the total sedimentation of the red cells at the end of sixty minutes expressed in millimeters.

Normally in men it varies from 0 to 8 mm., average 3 to 4; healthy women, 0 to 10 mm., average 5 to 6. In menstruation as high as 12 mm.

Wintrobe and Landsberg Method

Materials.—

Wintrobe tubes and venous blood collected in 3:2 oxalate mixture (see page 567).

Place the filled Wintrobe tube (see Fig. 226) in an exactly vertical position, at room temperature, and observe the point on the mm. scale to which the corpuscles fall during one hour.

The **normal average** sedimentation in one hour by this method is 3.7 mm. for healthy men, and 9.6 mm. for women, with a maximal range from 0 to 9 mm. for men and 0 to 20 mm. for women.

Comment: For many years it was recommended that the sedimentation rate be corrected for the volume of packed red cells. Wintrobe now feels that this adjustment is an unnecessary refinement of the procedure.¹

Landau Method²

Modification of Linzenmeier-Raunert Method

Before using, the sedimentation pipettes should be well cleaned with alcohol and ether and should be dried thoroughly in an oven or other warm place. If they are not completely dry, sodium citrate solution should be drawn up before the blood test is taken.

Insert capillary pipette into a precision syringe.

Draw up 5% sodium citrate solution by turning the top screw to the left until the upper meniscus of the solution has reached the lower mark *A*.

Lance finger tip or ear lobe of the patient deeply enough to get an even flow of blood without squeezing.

Draw up the blood until the height of the liquid reaches the upper meniscus of the upper graduation *B*, and try to avoid the formation of air bubbles rising in the blood solution. If bubbles should form during the test, they can easily be expelled by means of the top screw. The suction of the blood sample can easily be performed with one hand, by holding the syringe with the capillary pipette in dagger fashion with the middle, fourth and small fingers and manipulating the top screw with thumb and index finger.

Clean the tip of the capillary pipette with ether and continue to draw up the mixture into the bulb (by turning top screw to the left) until the lower meniscus of the blood column ends just a few millimeters below the lower opening of the bulb. (Do not draw blood into bulb completely.) The pipette may then be carefully shaken.

Force the blood up and down twice very slowly by turning screw to the left or right, respectively, in order to obtain a better mixture in the bulb. Force the blood gradually back into the capillary tube, close lower opening with thumb and hold pipette firmly while turning the syringe carefully, exerting a slight pull in order to lift out the capillary pipette which is then placed into the metal stand.

The blood mixture may hang at any place in the tube but should not touch the bottom. Citrated blood should always be drawn slowly into the capillary tube, otherwise part of it will cling to the walls and air bubbles will form. If air bubbles have formed in the blood column, turn syringe upside down at an angle and force out the air bubbles by turning the screw to the right. This will push the blood column toward the opening of the pipette and thereby expel the air bubbles without the loss of much blood.

The instrument consists of a metal stand which holds either six or twelve capillary pipettes, 12 cm. long with an inside diameter of 1 mm. Each pipette is provided with two graduation marks, one at 12.5 and the other at 62.5 mm. above the tip. The pipettes are

¹Wintrobe, M. M.: Personal communication, November, 1954.

²Landau, A.: *Am. J. Dis. Child.* 45: 691, 1933.

held in place by strong bottom springs which press the pipette against corresponding cavities provided in the screws, which are fitted into the upper part of the metal stand directly vertical above the bottom springs.

To insert the pipettes into the stand, press down with the tip of the pipette against the bottom spring, and while holding it down, insert the upper part of the pipette into the cavity, by gradually releasing the pressure on the spring. In the upper part of the pipette is a small bulb similar to the bulb in an ordinary blood pipette.

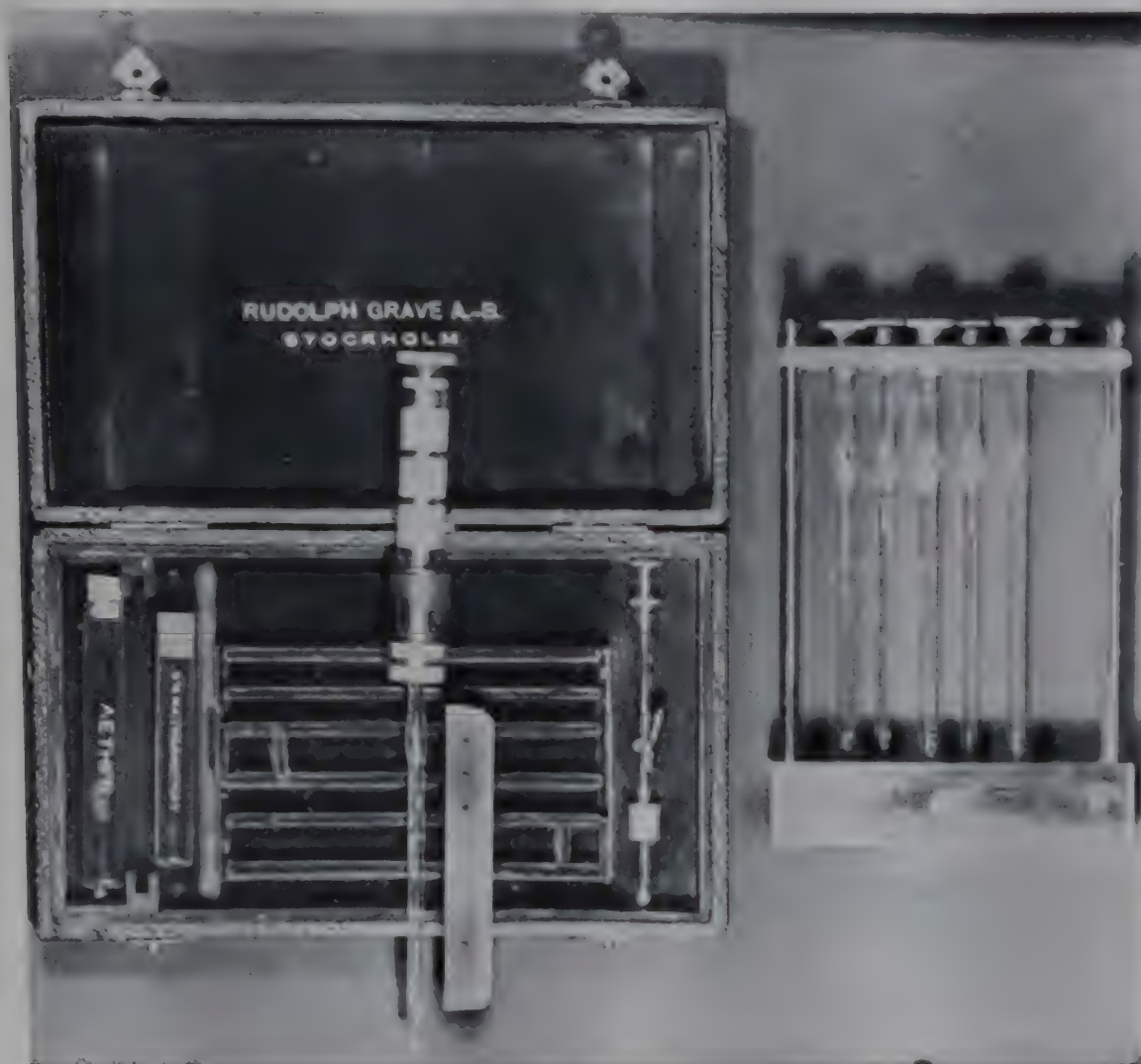


Fig. 228.—The Landau modification of the Linzenmeier-Raunert blood sedimentation outfit.*

The precision syringe consists of a metal barrel with ground-in plunger which is adjusted by means of a screw. The lower part of the barrel has a perforated rubber stopper fitted inside into which a capillary pipette can be inserted. By adjusting the bottom screw, the size of the perforation of the stopper can be increased or decreased. Near the bottom, the barrel of the syringe has a fine opening in the side which ordinarily is covered with a piece of rubber tubing. Before inserting the capillary pipette, which already has been filled with citrated blood, into the syringe, it is necessary to roll upward with the thumb the pointed part of the rubber tubing and thus expose the side opening of the metal cylinder ordinarily covered by the tubing. By covering the tip of the capillary pipette with the finger, the pipette may be inserted into the syringe as far as it will go, and then the rubber tubing released. In keeping the side opening of the pipette exposed, an excess of pressure in the syringe can be avoided which would tend to expel the blood. In the event that the capillary pipette has a very small outside diameter, the rubber stopper in the syringe may have to be exchanged for one with a smaller opening. If the plunger should fit too tightly, a drop of oil, dropped into the cylinder's upper part, will usually make it slide smoothly again.

The metal housing with a capacity of six capillary pipettes is protected by a velvet-lined case which also holds glass vials for ether and 5% sodium citrate solution and automatic

*This may be procured from Clay-Adams Co., Inc., 44 East 23rd St., New York, N. Y.

blood lancet, rule, rubber tubing, compartment for the precision syringe and room for cotton and compressors. The stand, together with glass capillary pipettes, can be placed in horizontal position in the case.

Results.—

Readings are to be made in millimeters after one hour. The original length of the blood column is 62.5 mm.

Because of the length of the bulb, when using the precision syringe, only very little citrated blood will cling to the walls of the capillary pipette; thus the definite length of the column would deviate only one or a few millimeters at the most from the original length, and this small difference would be of no practical importance. One can also determine the length of the column as approximately 60 mm.

Normal Values.—For children under 2 years, and for men, from 1 to 5 mm. per hour. For children over 2 years, and for women, from 1 to 8 mm. per hour. Slightly increased values are from 6 to 14 mm. and from 9 to 14 mm., respectively. Fairly high values are from 15 to 25 mm. High values are over 25 mm.

The Landau modification of the Linzenmeier-Raunert method of sedimentation is especially useful in pediatrics since it does not entail venepuncture. Venepuncture in children is often attended with difficulties; therefore, any method which avoids it is to be recommended.

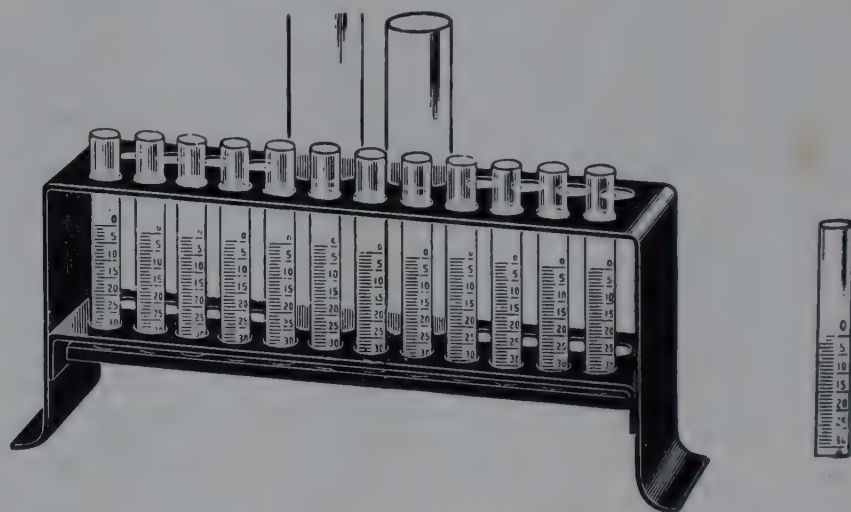


Fig. 229.—Walton rack and tubes. (Hellige, Long Island City, N. Y.)

Materials.—

Walton Modification² of the Cutler Method

Tubes 60 mm. long. The internal diameter is such that a tube contains 1 c.c. within the graduated portion, which is divided from 0 to 32.5 mm. in 0.5 mm. divisions, with every tenth line numbered.

3.7% solution of sodium citrate.

5 c.c. syringe.

Technic.—

Exactly 0.5 c.c. of a sterile 3.7% solution of sodium citrate is drawn into a clean sterile 5.0 c.c. syringe.

Syringe is filled to the 5.0 c.c. mark with venous blood from the cubital vein.

Empty syringe into small, clean, dry tube, and gently mix with citrate by inverting a few times.

If patient's erythrocyte count is between 4,500,000 and 5,000,000 no correction need be made. Draw the blood from the vial to the 20 mm. mark on the sedimentation tube and place in upright position.

²Walton, A. C. R.: J. Lab. & Clin. Med. 18: 711, 1933.

Correction of this procedure with variations in the red cell count: If count is above 5,000,000, plasma must be added; if the count is low, plasma must be removed. The amount to be added or removed can be determined by the following formula:

$$\frac{X}{Y} - 1 = \text{amount of plasma to be added to (or removed from) 1 c.c. of blood.}$$

X = number of million cells per cubic mm.

Y = desired number of million cells per cubic mm.

Thus if a patient has an erythrocyte count of 4,000,000 the calculation would be

$$\frac{4.0}{5.0} - 1 = - 0.2 \text{ c.c.}$$

or 0.2 c.c. of plasma per 1.0 c.c. of blood would be removed after centrifuging down the cells. If a count should be 5,400,000 the calculation is

$$\frac{5.4}{5.0} - 1 = + 0.08 \text{ c.c.}$$

or 0.08 c.c. of plasma should be added to each c.c. of blood. The plasma is obtained by centrifuging about 1 c.c. of blood and pipetting off the required amount of plasma.

At the end of one hour the amount of sedimentation is measured from the bottom of the meniscus of the supernatant plasma to the top of the red cell column.

Results.—

Normal sedimentation rate is never above 8 mm. During menstruation women may have a sedimentation rate of 8-12 mm. During pregnancy the rate may go to 14 mm. In the absence of menstruation or pregnancy a rate of over 8 mm. indicates the existence of a pathologic condition.

Balachowsky Method

This is called the sedio-tassometer of Balachowsky. The instrument is designed for ten or less simultaneous determinations. It is composed of a frame bearing the pipettes in spring clips. The frame rests on a support provided with four adaptable screws and a water level to assure absolute perpendicularity. The frame revolves on an axis and can therefore take two positions: the horizontal position and the vertical or working position. Different types of pipettes are furnished (a) one with 100 mm. graduation, reading the sedimentation in millimeters; (b) with six marks between 18 and 36 millimeters; (c) without any division, taking the readings with a centimeter tape.

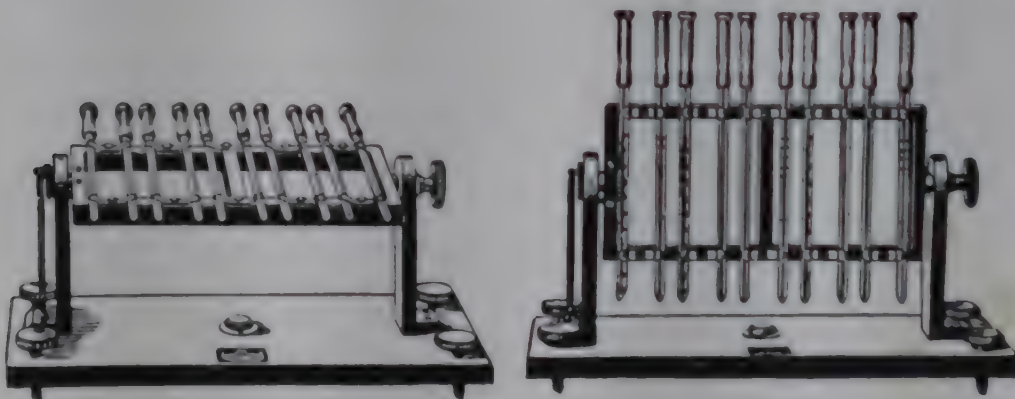


Fig. 230.—Balachowsky blood sedimentation apparatus (Heilige, Long Island City, N. Y.).

The top part of each pipette is provided with a suction tube composed of two parts: a rubber muff permitting the filling of the pipette with blood and to raise its upper level to the desired height and a suction screw embodied in the rubber muff, with which to bring the level of the blood column to precisely the required point "1" of the graduation, thus making the speed reading very easy.

Technic.—

Test the rocking frame, the suction screw, and rubber muff. Rinse a pipette with 5% neutral solution of potassium oxalate. Expel this fluid and take up the blood from a finger prick, avoiding air bubbles. Place pipette on the frame in the first position; fill as many more pipettes as is necessary to make the requisite number of tests. Compress and release slowly the suction tubes on the pipettes in order to allow the blood its full homogeneity. Turn the frame into a vertical position and note time. After 20 and 30 minutes read the sedimentation rate per hour. Do not use any pressure on finger in taking the blood.

Rourke-Ernstene Method¹

Normal Values: 0.05 to 0.40 mm. per minute.

Material Required.—

Sedimentation tube 120 mm. long with 2 mm. divisions from 0 to 100 mm. Internal diameter of tube, 4 mm. The Wintrobe tube may be substituted here.

Blood sample collected in heparin or "3:2" oxalate mixture.

Technic.—

Fill the sedimentation tube with the blood sample to the 0 mark.

Place the tube in a suitable rack in a vertical position.

Take 6 to 10 readings at regular intervals, until the readings indicate marked slowing in rate of settling of the erythrocytes.

Calculation.—

The calculations may be made either by using the data as recorded or by preparing a curve. Take the figures only during the period of *fastest* rate of fall, which usually eliminates the first 10 to 15 minutes, and the later period of observation.

Divide the total fall in mm. during the fastest period of fall by the time required for the fall. Express the result in mm. per minute.

Example.—

Time in Minutes	5	10	15	20	25	30	35	40	45	50	55	60
Readings in mm.	2	3	6	9	15	22	29	34	36	38	40	41

The fastest fall is between 20 and 40 minutes, 25 mm.

$$\text{Sedimentation index} = \frac{25}{20} = 1.25 \text{ mm./min.}$$

Summary of the Pertinent Facts on the Sedimentation Test**Normal Values.—**

Westergren: Men: 0 to 15 mm. after one hour

Women: 0 to 20 mm. after one hour

Linzenmeier: Men: 350 to 600 minutes

Women: 300 to 600 minutes

Under 200 minutes is pathologic

Cutler: Men: 0 to 8 mm. after one hour (3 to 4 mm. in one-hour average)

Women: 0 to 10 mm. after one hour (5 to 6 mm. in one-hour average)

Wintrobe and Landsberg: Men: 0 to 9 mm. in one hour (3.7 mm. average)

Women: 0 to 20 mm. in one hour (9.6 mm. average)

Landau: Men: 1 to 6 mm. in one hour

Women: 1 to 9 mm. in one hour

Walton: Men and women: never over 8 mm. in an hour.

Comments on Sedimentation Rate Determination.—

Peightal² very properly believes that the test is not a specific diagnostic sign for any disease, but indicates the presence or absence of an inflammatory process. In some conditions it may be used in part to estimate the severity

¹Rourke, M. D., and Ernstene, A. C.: *J. Clin. Invest.* 8: 545, 1930.

²Peightal, T. C.: *New York J. Med.* 36: 173, 1936.

of an infection. In diseases of the pelvis a careful history and physical examination are necessary to exclude the presence of infection elsewhere; the examiner must remember that infected sinuses and teeth as well as acute rheumatic fever, tuberculosis, etc., cause rapidity of sedimentation. Pregnancy increases the rate very much. The increase is gradual from the third month until the ninth month, when it may be five times the average normal. This change in rate disappears four weeks post partum.

It is in the differential diagnosis of conditions causing pain in the right lower quadrant that the sedimentation test furnishes a very definite aid, particularly within the first forty-eight hours of the onset of symptoms. Grodinsky, Lesser and Goldberger, and Smith have shown that the sedimentation rate remains approximately normal in acute appendicitis for at least the first forty-eight hours from the onset of symptoms, and during the catarrhal, suppurative, and even gangrenous stages. Increased rates are observed only in localized abscess or general peritonitis. In cases of acute salpingitis, before one recognizes the swollen tube by bimanual examination as a palpable mass, only tenderness and spasm being revealed, the sedimentation rate is markedly increased (50 to 100 mm. in an hour). Thus in this test there is a factor in differentiation between acute appendicitis and early acute salpingitis. It must be emphasized that when the disease process produces a palpable mass, one cannot distinguish between appendiceal and tubal conditions just by the sedimentation rate, for abscess of the appendix may give a rate equal to that of the infected tube. If, however, abscess of the appendix can be ruled out in any case of pelvic mass, then the sedimentation test becomes an important adjunct in ascertaining the nature of that mass. With regard to salpingitis, unruptured ectopic pregnancy, twisted ovarian cyst, hemorrhage into a large graafian follicle or cyst, and painful ovarian swelling related to ovulation, one must consider the history and pelvic examination by far the most important factors in making the correct diagnosis, while temperature, blood count, Aschheim-Zondek test, and sedimentation rate only help to influence the opinion. A swollen inflamed tube may show a sedimentation rate of from 50 to 100 mm. and a time of 30 to 50 minutes, while an unruptured ectopic pregnancy with very little or no blood in the peritoneal cavity will have a rate between 20 to 35 mm. and a time of 100 to 120 minutes. A twisted ovarian cyst may cause a very high sedimentation rate if there has been much interference with the circulation of the tissue, while hemorrhage into the follicle or cyst and painful ovarian swelling usually cause no increase in the rate.

The test has been of great benefit in prognosticating the degree of healing in salpingitis or postabortional infections and in indicating the proper time to operate. Previously, dependence was placed upon the temperature curve and leukocyte count for this information. Consequently, patients with pelvic infection were allowed to get out of bed too soon and had relapses, or operations were performed in the "too acute" stage. It was found that the temperature curve and leukocyte count return to normal often many days before the sedimentation rate has been materially reduced. By keeping patients with acute salpingitis in bed until the sedimentation rate falls below 30 mm. in an

hour, there is practically no relapse. The same figure is used in deciding upon operability of chronic tubal disease. This avoids abdominal operation in the "too acute" stage, and postoperative convalescence is far smoother.

Landau,¹ in discussing the usefulness of microsedimentation rate in pediatrics, emphasizes its employment in arthritic and tuberculous conditions. In a number of cases, the test was useful in children, bedfast for a long time, whose primary illnesses during their stays at the hospital were followed by regularly made microsedimentation tests. In such cases an increase in sedimentation rate occurred as the symptom of a complication several days before a rise in temperature occurred. Usually the microsedimentation decreased much later than the temperature fell, sometimes reaching the desired value only several weeks after the temperature had become normal. Westergren considers pronounced sedimentation to be characteristic of acute polyarthritis. High values in light cases of acute polyarthritis are important as an indicator of the intensity of the process of the disease. Not until sedimentation has reached normal values after repeated tests can one regard the infectious process as having run its course. Since, apparently, rheumatic illnesses are very common in Gothenburg, Sweden, Landau had numerous opportunities of testing this method in such cases. He found it quite useful in following the progress of acute polyarthritis, acute endo-, peri-, and myocarditis, and in chorea. He has found this test very helpful in facing this important question: When, after the temperature has subsided to normal, can one venture to pronounce the endocarditic process ended in acute endocarditis? In other words, when does the endocarditis end and the vitium, the resultant condition, begin? This also raises the important question of the continued therapeutic regimen, particularly physical therapy, as well as the prognosis for cardiac function. Landau states that, in addition to cases in which the diagnosis is doubtful, microsedimentation has proved to be of extraordinary value as an indicator of disease, being generally far more sensitive than the body temperature. In routine treatment of acute polyarthritic and cardiac diseases at the Gothenburg Hospital for children, it was his custom not to release patients from bed until microsedimentation had shown normal values after repeated tests, irrespective of whether or not the body temperature had become normal. We do not agree that such rigidity in management is necessarily wise and that return to activity can be judged by the sedimentation test *alone*. The *total* clinical picture must be taken into account for such decisions.

In a study of 25 cases of chorea it was found that when chorea had existed as the only rheumatic condition, or in which polyarthritis or endocarditis had preceded it so long before that chorea could not be considered as having arisen in direct connection with these diseases and in which complicating commonplace infection could be precluded, repeated microsedimentation tests showed low values within the limits of normal. On the other hand, in cases in which the chorea arose at the same time as, or in direct connection with, polyarthritis, endocarditis, or pericarditis, microsedimentation was increased, and the sedimentation curve seemed to be influenced by the other diseases. It was upon

¹Landau, A.: *Am. J. Dis. Child.*, 45: 691, 1933.

this finding that Landau based his agreement with Vollenberg's hypothesis of chorea as a metarheumatic disease, or as an intoxication of the cerebral cortex.

The blood sedimentation rate has been used extensively in following patients with pulmonary tuberculosis as a gauge of activity and response to therapy. In tuberculosis of the bronchial lymph nodes in children during periods of activity the blood sedimentation rate is elevated. Westergren states that blood sedimentation in bronchial lymph node tuberculosis in children after one hour is 50 mm. during the period of activity. It then sinks rapidly and after two months is around 10 mm. Dehoff found that in active tuberculosis of the hilus the blood sedimentation rate with the Linzenmeier tube was such that 280 minutes were required for the blood plasma to reach the mark 18 mm. Rókay, using the Westergren method, found the blood sedimentation values as given in Table 82, in cases of hilar gland tuberculosis.

TABLE 82

SEDIMENTATION RATE IN MM.	NUMBER OF CASES	CLASS I*	CLASS II	CLASS III
0— 3	2	—	2	—
4— 6	7	2	4	1
7— 12	16	6	4	6
13— 17	1	—	1	—
18— 25	3	1	2	—
26— 37	3	—	2	1
38— 50	1	—	—	1
51— 70	—	—	—	—
71— 89	—	—	—	—
90—120	1	—	—	1

*Class I, slightly enlarged glands; Class II, glands size of hazelnut; Class III, glands size of hen egg.

In Rókay's second table the activity of the tuberculous process is compared with the blood sedimentation.

TABLE 83

SEDIMENTATION RATE IN MM.	CASES	ACTIVITY		PROGNOSIS		
				GOOD	DUBIOUS	BAD
		0	+			
0— 3	2	2	—	1	1	—
4— 6	7	5	2	7	—	—
7— 12	16	10	6	9	7	—
13— 17	1	—	1	—	1	—
18— 25	3	—	3	—	3	—
26— 37	3	—	3	—	1	2
38— 50	1	—	1	—	—	1
51— 70	—	—	—	—	—	—
71— 89	—	—	—	—	—	—
90—120	1		1	—		1

Blood sedimentation in children with polycythemia is very high whereas in adults it tends to be low. According to Schönberger,¹ in myxedema the blood sedimentation rate is enormously accelerated, but glandular therapy may return it to normal very rapidly.

¹Schönberger: Ztschr. f. Kinderh. 38: 688, 1924.

In arthritis in adults, the sedimentation rate is definitely increased in the infectious or atrophic, rheumatic and rheumatoid type, and is normal in the noninfectious, or hypertrophic, degenerative, osteoarthritic type.

In psychoneurotic cases with unusual symptoms, in which no organic lesion may be found, repeatedly normal sedimentation rates aid in excluding organic disease.

The sedimentation rate is retarded in asthma, hay fever, and urticaria and other allergic states.

In pertussis, with the sedimentation rate normal and a high lymphocyte count, we have an unusual condition, namely, a normal rate in a febrile disease. In all other febrile diseases, it is apparently increased.

It must be borne in mind that the sedimentation rate and the hemogram are both independent of the fever curve. They both may be negative or positive with and without fever, but they parallel temperature reactions only in the climax of severe inflammatory processes. Expressed in other terms, the hemogram reaction is exceedingly useful in acute infections, while the sedimentation rate is important in the more chronic conditions.

The sedimentation rate has been found useful in following the course of coronary occlusion in adults. The rate tends to be high early and increases as the myocardial infarction spreads. Decline in the rate occurs when the process of infarction ceases. In other cardiac diseases in adults not associated with infarction, the sedimentation rate tends to be normal.

In summary, the sedimentation rate finds a wide field of clinical applicability. It is not a specific diagnostic test, although it may influence differential diagnosis. Its greatest usefulness is in following the course of diseases known to have increased sedimentation rates. As an adjunct to its reflection of the progress of such diseases, the sedimentation rate acts as a yardstick of the efficacy of treatment. Although single determinations have value, *changes* in multiple determinations in a given case are of even greater significance in the evaluation of a patient.

Methodology in sedimentation rate determinations deserves some comment. The various types of apparatus and the dissimilarity of reporting results attest to the interest manifested in this procedure. In general, we feel that the test has been made unnecessarily complicated, considering its clinical usefulness. Any method with one reading in an hour, giving the amount of settling of the erythrocytes, appears quite adequate. Those methods requiring graphs of various types appear to us as unnecessary refinements. We have included many of the methods in this text for historic interest rather than clinical value.

Greisheimer, Treloar, and Ryan² attempted to draw conclusions about the average relationship existing between the Cutler, the Linzenmeier, and the Westergren methods. They found that a sedimentation of one hour for normal subjects seems to be reasonably concordant for the three methods despite the wide differences in tube width, anticoagulant concentration, and length of fluid column, although the differences between the means for the three methods are significant statistically. The average sedimentation at one hour for women is approximately double that for men. They showed by tables the

²Greisheimer, E. M., Treloar, A. E., and Ryan, M.: *Am. J. M. Sc.* 187: 213, 1934.

various indices and differences between the three methods and attempted to establish some ratios between them. Their tables demonstrate that the regression lines for predicting the most likely value to be expected by any one test when that by another is known are of three distinct types. There is a rectilinear relationship between the Cutler index and the Linzenmeier index. The linear regression differences between Westergren index and Cutler or Linzenmeier index appear to be somewhat like saturation curves. It was impossible to fit this into a systematic scheme by any type of mathematical equation. The relationships between the various indices and Linzenmeier time values may be suitably portrayed by sections of hyperbolae. Individual tables have been given by them for each independent variable of clinical interest. The dispersals of individual cases about the lines of average relationship, although not studied in detail, are clearly greater than those ascribable to errors inherent in the technics. It is obvious that sedimentation measures for human blood are, in part, specific for the technic employed. It would be advantageous, therefore, to have a standardized method for blood sedimentation for clinical work but, as yet, this has not been done. We must, therefore, in estimating the importance of the sedimentation rate in any individual case be familiar with the sedimentation rate in such cases, the method used, and the normal value for that method.

Watkins¹ emphasized the fact that variations in the room temperature markedly affect the sedimentation rate. Sedimentation rate is retarded in a cooler room. Table 84 gives comparisons on stored blood and blood rechecked after 18 hours.

TABLE 84

CASE	ROOM TEMPERATURE		AFTER 18 HRS.		
	READING		AT 10° C.		
1	24 mm.	(1 hr.)	3 mm.	(2 hrs.)	5 mm. (3 hrs.) 9 mm.
2a	31 mm.		15 mm.		
3b	27 mm.		25 mm.		
4	24 mm.		17 mm.		
5c (25° C.)	12 mm.	(37° C.)	20 mm.		

a. Hemolytic anemia.

b. Pregnancy in latter months.

c. The variation between 25°C. and 37° C. in the sedimentation rate of erythrocytes is exactly 8 mm. There is a change from a normal or approximately normal to a definitely increased sedimentation rate of erythrocytes.

In carrying out blood sedimentation tests, either an electric light bulb or preferably the standard bacteriologic incubator can be used quite satisfactorily. Racks containing the blood can be placed near the lamp or in the incubator. A thermometer must be placed near the blood so that records of the temperature can be made quickly and accurately. Watkins preferred the small Cutler tube for this work because (1) the smaller tube affords less surface area exposed to the reducing of temperature; (2) smaller amounts of blood can be used; (3) it is less cumbersome in handling and placing in the incubator; and (4) it maintains constant temperature.

It is important to hold the blood near body temperature until the test can be recorded. Chilling the blood before making the test will definitely alter the final results.

The influence of anemia on the blood sedimentation rate has been studied by many hematologists. Certain authors have suggested a correction of the

¹Watkins, R. G.: Lab. Digest 10: 4, 1946.

sedimentation rate taking into consideration the number of erythrocytes, the blood volume, the corpuscular volume, the average diameter of the erythrocytes, or their hemoglobin content.

According to Adura,¹ the influence of the erythrocytes on the sedimentation rate, if present at all, is very small *in vivo*. Therefore, for actual practice, consideration of the erythrocytes is unnecessary. The essential factors in sedimentation of erythrocytes are the physicochemical and electric alterations of the plasma. Specific weight of the erythrocytes is not to be considered a major factor. Adura points out that the sedimentation rates differ greatly in cattle and horses, although the specific gravity is identical. Comparative studies made on hypochromic and hyperchromic erythrocytes showed that hemoglobin content does not materially influence the sedimentation rate. It can be shown, also, that the average diameter of the erythrocytes is of no significance in this respect. Adura pointed out that when erythrocytes from pregnant women or from patients with infectious diseases (accelerated sedimentation) were washed and then placed in the plasma of normal persons, a normal sedimentation rate was obtained. On the other hand, when the washed erythrocytes of healthy individuals were placed in plasma of pregnant women, the sedimentation rate was greatly accelerated.

We are in agreement with Adura that these experiments prove that the plasma is the regulating factor in blood sedimentation, and that correction of the sedimentation rate on the basis of factors concerning the erythrocytes may even diminish the value of the reaction, as thereby an extrinsic and inconstant factor is introduced which unnecessarily complicates the otherwise easy test.

The comments in the preceding two paragraphs have appeared in previous editions of this text. Wintrobe, who has been a strong advocate of correction of the sedimentation rate for anemia, now has abandoned such corrections as an unnecessary refinement of a crude test. Note of his present stand under the Wintrobe technic has been made, and the correction chart which formerly accompanied his technic has been eliminated. Meyers and associates² tried to devise some means of adjusting all sedimentation rates to a standard cell volume and as a result of their studies cast "serious doubt on the validity of any such correction charts."²

HEMATOLOGY OF INFANTS AND CHILDREN

Since blood pictures in infants and children normally differ somewhat from those in adults, we shall give briefly the salient differences so that proper interpretation of findings may be made.

At birth the number of white cells is very high, around 30,000, declining to normal after the first week. It is also interesting to note that there is a left shift during the first week of life. The count of the blood platelets is also variable during the first week, sometimes reaching very high proportions.

The percentage of hemoglobin in blood at birth is very high, figures as high as 130 per cent having been obtained. Hemoglobin falls soon after birth, reaching normal around the end of the first week.

¹Adura, Ac.: *Merrame-Anais paulistas de medicina e cirurgia* 42: 135, 1941; *Internat. Med. Digest* 39: 6, 1941. W. F. Prior Co.

²Meyers, A. J., and others, *loc. cit.*

The number of red cells is very high at birth, beginning to fall on the third day after birth.

At birth, the presence of nucleated red cells in the film has no pathologic significance. The same is true of the great prevalence of reticulocytes and polychromatic cells. Anisocytosis is common and not pathologic.

The newborn child shows in its blood the last remnants of the nuclear structures of embryonal life. They persist up to the third or fourth day. The leukocytic system develops in the same way, phylogenetic lymphoid elements and genuine lymphocytes constituting the predominant element, followed by the appearance of lympholeukocytic and granulocytic elements. Naegeli, Schridde, and others maintain that lymphocytes develop embryonally very late, then steadily increase together with a general development of granulocytes and erythrocytes in the liver, spleen, kidneys, vascular system, and interstitial tissue. The bone marrow of the baby contains many more cells than that of the adult. This bone marrow is transformed into the fat marrow of the long bones, remaining hematopoietic, however, in the short bones. The

TABLE 85.—FRANK'S QUALITATIVE CHART OF NEWBORN INFANTS' BLOODS*

CASES	WHITE BLOOD CELLS	NEUTRO- PHILES	LYMPHO- CYTES	MONO- CYTES	TRANSI- TIONALS	EOSINO- PHILES	BASO- PHILES
12 hrs. after birth	29,000	71.2%	18.8%	0.7%	8.5%	0.8%	—
2 days	19,100	63.8%	30.2%	0.3%	4.5%	1.2%	—
3 days	13,300	50.0%	41.4%	0.8%	6.4%	1.4%	—
4 days	8,900	31.6%	57.6%	0.9%	9.6%	0.7%	—
5 days	12,000	34.3%	55.7%	1.3%	8.4%	0.3%	—
6 days	10,700	29.2%	60.5%	1.5%	8.3%	0.5%	—
7 days	10,400	29.2%	62.8%	1.0%	6.2%	0.8%	—
8 days	10,700	28.6%	63.2%	1.2%	6.2%	0.8%	—

*Baar and Stransky: Die klinische Hematologie des Kindesalters, p. 6, Franz Deuticke, Wien, 1928.

ratio between white blood corpuscles and erythrocytes is very much higher in the later embryonal months than in the adult. We know that there is a greater lability of the bone marrow of the child than of the adult. This is true of their lymphatic systems as well. There are far more unusual cells in the blood—plasma cells and atypical lymphocytes. It is, therefore, necessary in all cases to make intensive detailed studies of blood pictures of children in order to correlate them properly with clinical facts.

One must divide these young subjects into three groups in order to get a proper estimation of hematologic data: newborn babies; nursing infants; and infants from one to six years of age. The average adult blood picture is not reached before the sixth year, but even then, and sometimes later, lymphatic reactions are produced much more easily than in the adult and wide fluctuations are commonly seen. In general, the blood picture at birth is markedly neutrophilic and shows a great shift to the left. Physiologic myelocytes and juveniles have been seen at this time under perfectly normal conditions. The neutrophilia with marked shift has been explained by Zange-meister and Missl as the result of the influence of maternal blood or, as Arneth says, it represents a "pathologic" leukocytosis of the birth process. It is very probable that the accommodation of the body to the new circula-

TABLE 86.—BLOOD PICTURE IN NURSINGS AGES ONE TO TWELVE MONTHS*

NO. CASES	AGE IN MONTHS	HEMO- GLOBIN PER CENT	RED COUNT IN CU. MM. (MILLIONS)	WHITE COUNT IN CU. MM.	NEUTRO- PHILES PER CENT	I PER CENT	II PER CENT	III PER CENT	IV PER CENT	V PER CENT	EOSINO- PHILE PER CENT	BASO- PHILE PER CENT	LYMPHO- CYTE PER CENT	MONO- CYTE PER CENT	METAMY- ELOCYTE PER CENT
9	1	96	5.51	16,130	24	8	40	36	11	5	2.0	1.5	60	11.0	0.5
8	2	96	5.5	16,040	24	10	43	31	10	4	2.0	1.5	61	10.5	1.0
8	3	96	5.43	13,100	24	11	44	28	13	4	2.5	1.0	62	10.0	0.5
7	4	95	5.41	13,000	23	10	37	36	15	2	2.5	0.5	62	12.0	-
8	6	95	5.39	12,850	21	8	42	35	11	4	2.5	0.5	64	12.0	-
6	8	94	5.36	12,600	24	9	41	40	7	3	2.5	0.5	62	11.0	-
7	10	93	5.35	12,140	27	8	43	36	9	3	2.5	1.0	60	9.5	-
6	1	95	5.22	16,600	26	10	39	36	15	6	2.0	1.0	59	9.5	2.5
6	2	93	5.15	16,430	29	9	42	31	13	5	2.0	1.0	57	8.5	2.5
5	4	93	5.14	12,700	32	11	44	32	10	3	2.0	0.5	54	10.0	1.5
6	6	91	5.07	12,100	34	11	41	31	12	5	1.5	0.5	53	10.0	1.0
7	10	90	5.04	11,540	36	12	40	34	10	4	1.5	0.5	53	8.5	0.5
7	12	90	5.01	11,100	37	10	39	30	15	6	1.5	0.5	53	8.0	-
8	1	91	5.2	17,210	27	9	36	34	10	5	1.5	1.0	56	11.0	3.0
7	2	90	5.14	17,260	30	11	39	33	13	4	1.5	1.5	54	10.5	2.5
6	4	88	5.03	12,200	32	12	40	34	10	4	1.5	1.5	52	10.5	2.5
7	6	88	4.95	11,600	36	12	43	32	10	3	1.5	1.5	49	10.0	2.0
6	10	84	4.9	11,000	40	14	45	30	9	2	0.5	0.5	48	9.5	1.0
8	12	84	4.83	10,110	42	13	42	34	8	2	0.5	0.5	47	9.0	0.5

*Baar and Stransky: Die klinische Hematologie des Kindesalters, Franz Deuticke, Wien, p. 42.

tion and respiration irritates the residue of embryonal blood forms and liberates them into the blood stream. Lymphocytes increase rapidly in the relative picture.

The blood pictures of prematurely born babies exhibit greater abnormalities than those of normally born children; that is to say, their blood pictures may closely approximate the embryonal blood picture. Prematurely born children at first show a high leukocytosis and neutrophilia. This recedes, together with the marked nuclear shift.

The Blood Picture of Nursing Infants

The blood picture of the nursling remains constant in the first year and up to the middle of the second; at this time, it begins to change gradually into an appearance like that of the blood of the adult, finally attaining this adult appearance about the sixth year. There is still a high *lymphocytosis* together with a slight nuclear shift under perfectly normal conditions. The number of neutrophiles in the nursing child is about 25 to 30 per cent and the lymphocytes are from 42.5 to 90.5 per cent, according to Hofmann and Welker. Gundobin considers this the average for nurslings: neutrophiles 34.6; lymphocytes 59.0; and monocytes 6.4.

Zibordi¹ reports the following investigation of breast-fed children, mixed-breast-and-artificial, and artificially fed children with the Arneth classification. In the first group are designated the breast-fed children up to the tenth month; in the second group, children on a mixed diet; and in the third group, artificially fed children.

Morphology of the Blood in Infancy

There is an extreme lability of the blood-forming apparatus in early infancy; that is to say, it is much more sensitive to irritation than that of the older individual.

TABLE 87

AGE	NO. OF CASES	GRAMS OF HEMO- GLOBIN	VOLUME PER CENT	PER CENT OF HEMO- GLOBIN	MAX- IMUM	MIN- IMUM
2-3 weeks	6	17.0	22.8	123	133	108
3-4 weeks	4	15.7	21.1	114	127	110
4-5 weeks	6	14.4	19.3	104	117	87
5-6 weeks	3	13.7	18.3	99	122	77
6-7 weeks	4	12.7	17.0	92	99	85
7-8 weeks	4	12.0	16.1	87	97	77
2-3 months	6	10.9	14.6	79	86	75
3-6 months	19	11.5	15.4	83	88	70
6-9 months	16	11.2	15.0	81	87	70
9-12 months	6	11.0	14.8	80	84	74
1-2 years	11	11.6	15.5	84	94	72

TABLE 88

AGE	POLYMORPHO- NUCLEARS	LYMPHO- CYTES	TRANSITION- ALS	LARGE MONO- NUCLEARS	EOSINO- PHILES
1- 6 months	34.54%	50.78%	10.52%	0.57%	3.59%
6-12 months	40.84%	49.21%	8.25%	0.94%	0.76%
1- 2 years	41.99%	47.0 %	7.52%	0.45%	3.04%

¹Zibordi: Ematologia infantile 1.z. Mailand, 1925.

TABLE 89

AGE	NEUTROPHILES	LYMPHOCYTES	MONOCYTES AND TRANSITIONALS	EOSINOPHILES
2- 4 months	29.1%	56.3%	11.2%	2.8%
4- 6 months	27.9%	56.9%	11.1%	3.9%
6- 8 months	28.1%	58.3%	9.8%	3.4%
8-10 months	28.0%	61.2%	8.2%	2.5%
10-12 months	33.7%	56.2%	7.1%	3.0%
1- 2 years	34.0%	54.5%	6.6%	5.0%

TABLE 90

AGE	HB. PER CENT	RED BLOOD COUNT	WHITE BLOOD COUNT	PLATELET COUNT
3 weeks	-	5.4 million	-	392,000
4 weeks	120	5.2 million	36,300	296,000
7 weeks	-	4.4 million	-	336,000
2 months	85	4.4 million	6,900	464,000
2 months	75	3.9 million	5,700	568,000
2 months	85	3.6 million	7,600	304,000
9 weeks	75	3.5 million	14,000	272,000
10 weeks	-	3.1 million	7,900	280,000
3 months	70	3.8 million	17,500	292,000
3 months	60	3.6 million	20,000	368,000
5 months	75	4.2 million	7,600	304,000
6 months	70	4.03 million	7,800	272,000
6 months	75	3.4 million	3,450	288,000
6 months	85	4.8 million	13,900	408,000
7 months	-	5.8 million	-	516,000
8 months	80	5.45 million	15,600	416,000
12 months	80	4.6 million	11,000	328,000

TABLE 91.—HEMOGLOBIN CONTENT IN CHILDREN

AGE	PERLIN	LICHTENSTEIN	WICK	WIDOWITZ	STIERLIN	CARSTANJEN
1- 2 years	78-80%	74-78%	-	62-110%	44-78%	70%
2- 3 years	80%	75%	-	62-110%	44-78%	76%
3- 4 years	80%	77%	83-110%	98%	44-78%	75%
4- 5 years	84%	77%	83-110%	92%	44-78%	75%
5- 6 years	81%	79%	-	86-110%	80.5 %	75%
6- 7 years	85%	80%	-	86-110%	80.5 %	65%
7- 8 years	85%	80%	-	86-110%	80.5 %	85%
8- 9 years	85%	79%	-	86-110%	80.5 %	73%
9-10 years	85%	79%	-	90-110%	80.5 %	70%

TABLE 92

AGE	POLYMORPHO- NUCLEARS	LYMPHO- CYTES	TRANSI- TIONALS	LARGE MONO- NUCLEARS	EOSINO- PHILIC LEUKO- CYTES
1- 2 years	41.99%	47.00%	7.52%	0.45%	3.04%
2- 3 years	48.23%	38.45%	8.72%	0.7 %	3.9 %
3- 4 years	52.63%	33.2 %	7.95%	0.48%	5.74%
4- 5 years	60.98%	25.08%	7.29%	0.35%	6.3 %
5- 6 years	55.39%	31.04%	6.85%	0.5 %	6.22%
6- 7 years	57.75%	30.28%	8.04%	0.59%	3.34%
7- 8 years	60.58%	27.98%	7.29%	0.46%	3.69%
8- 9 years	58.55%	27.98%	7.51%	0.43%	5.33%
9-10 years	56.99%	28.43%	8.73%	0.32%	5.63%
10-11 years	51.86%	33.03%	7.36%	0.44%	7.31%
11-12 years	60.67%	28.26%	7.54%	0.43%	3.1 %
12-13 years	53.79%	33.25%	8.81%	0.58%	3.57%
13-14 years	62.82%	25.88%	6.92%	0.22%	4.16%
14-15 years	56.45%	28.22%	8.87%	0.6 %	5.86%

Many authorities have noted the high percentage of hemoglobin and increase of cell count at birth. These figures rapidly fall after the first few days. Perlin noted a drop in hemoglobin after the fourth day with a gradual decrease, reaching its minimum at the end of the first year, namely, 58 to 78 per cent. From the second year on, it begins to rise, up to the fifteenth or sixteenth year of life.

The count of the red cells drops soon after birth. Toward the end of the first year, it is at its lowest point; namely, 4,200,000, when the count begins to rise up to the end of the fourth year.

Williamson and Appleton¹ give the figures shown in Table 87.

The number of red and white cells as given by Carstanjen² is shown in Table 88.

Karnicki gives the qualitative blood picture shown in Table 89.

There is a difference in the hemoglobin content and the number of red cells in breast-fed children and artificially fed children, about 10 per cent, according to Zibordi.

One must be careful in infancy cases in drawing conclusions from the shift of the neutrophils for the reason that 1.5 per cent juveniles and 18 per cent stabs as well as an occasional myelocyte have been found in healthy infants. This is due to the remarkable lability of the hematopoietic apparatus in infancy. *The hemogram of Schilling is useful in infancy but not from a single examination. Conclusions must be drawn only from repeated examinations.*

The number of blood platelets in infancy varies greatly. The average count is between 200,000 and 300,000. McClanahan and Mitarbeiter³ in a report on 68 blood examinations in infancy give the figures shown in Table 90 on blood platelets.

The hemoglobin content of the blood of children from 1 to 10 years is seen in Table 91.

The qualitative blood picture according to Carstanjen from the first to the fifteenth year is shown in Table 92.

Sedimentation phenomenon of the red cells in infancy is different from that of adults. In the Linzenmeier test tube in adult females, sedimentation drops to 18 mm. in 800 to 1,000 minutes, in males in 1,200 to 1,400 minutes. In newborn babies, the sedimentation rate of 18 mm. occurs in from 6 to 7 hours, but after the first month, the rate is 90 minutes; and in diseases such as syphilis and tuberculosis it is even faster. (See pages 959 ff.)

Blood Picture of Prematurely Born Children

The blood of prematurely born children offers an interesting study. In the first two weeks, the average percentage of hemoglobin is 105; in the third week, 93; and in the fourth week, 68; in the second month, 55; in the third month, 47; in the fourth month, 50; and in the fifth month, 52. The number of red cells begins to decrease after the second month, reaching its minimum in the third month and attaining a normal figure in the sixth month. There

¹Quoted by Baar and Stransky: l. c.

²Carstanjen: Jahrb. f. Kinderhk. 52: 215, 333, 648, 1900.

³Arch. Pediat. 33: 757, 1916.

is a leukopenia in all these babies up to the sixth month. Lichtenstein regards the anemia of prematurely born babies as physiologic.

Frankenstein and Stecher, in a study of the blood picture of prematurely born babies for the first twelve weeks of life, show a lymphocyte average of 72 per cent in the second week, and 82 per cent in the fourth week, which drops later to 72 per cent. Segmented forms in the first five days are between 20 and 28 per cent, rising to 35 per cent, dropping to 26 per cent on the eighth day and to 10 per cent in the fourth week. The "stabs" decrease in the first week from 12 to 4 per cent. Juvenile forms in this period show 0.5 to 1 per cent.

Blood Picture of Children from One to Six Years

These subjects show a gradual approach of the nursling's blood picture to that of the adult. Note Table 92, page 978, of Carstanjen, showing the changes from the first to the fifteenth year. The decrease in lymphocytes begins definitely about the end of the second year.

Changes in the Leukocytic Picture Under Physiologic and Pathologic Influences in Infancy

Virchow first called attention to digestion leukocytosis. He noted an enlargement of the mesenteric lymph glands after a meal, together with an increase in white cells in the circulating blood. Gregor in 1898 failed to confirm this phenomenon in healthy infants. In eleven cases he found from three to five hours after a meal some degree of leukocytosis, but in six cases he noted a leukopenia. In general, digestion leukocytosis is not constant in infancy. On the contrary, there is more likely to be a leukopenia. Schiff and Stransky claim that in healthy, as well as in artificially nourished infants, whether the lymph is disturbed or not after a milk meal, there follows a leukopenia.

No reliable data are at hand as to the shift in the hemogram as a result of the digestion of food.

Wernstedt¹ found the leukocyte count in infants lower while asleep than it was when they were awake. There is a lymphocytosis described as a result of efforts in crying, but this disappears after thirty minutes of rest. It is probable that the lymphocytes in crying infants, and in whooping cough cases, come from the spleen and the thoracic duct. This exercise leukocytosis was first described by Grawitz in 1910.

In intoxications of infants, not due to infectious diseases, the total white cell count may be elevated up to 20,000. There is a slight shift to the left in such cases. In intestinal decomposition, there is a slight shift, and in extreme cases of marasmus, there is a definite left shift. In general, however, a left shift speaks for an infection. In acute toxicosis, there is a leukocytosis.

The significance of eosinophilia in infancy has been the subject of much discussion. In general, we find an eosinophilia in all cases of exudative diathesis. This is particularly true of eczemas of infants, but it is believed that eosinophilia is not due to the local process, but to the underlying cause of

¹Monatschr. f. Kinderh. 9: 343, 1911.

eczema. Figures as high as 65 per cent eosinophiles have been noted. Eosinophiles in all acute dermatoses are decreased, and they are increased in all chronic cases, particularly in eczema. In erythrodermia desquamativa, the leukocyte count is elevated to 12,000 with a high-grade anemia and with a count of eosinophiles between 1 and 6 per cent.

Erythroblastosis Fetalis (Icterus Gravis Neonatorum)

This is often a fatal condition which has been the subject of considerable investigation. It is usually due to an intra-group hemolytic reaction incident to the mating of an Rh-positive father with an Rh-negative mother. (See pages 1026 ff., 1031 to 1055.) These cases must be clearly distinguished from icterus of the newborn due to congenital syphilis. It has been well described clinically by Buhler, Seely, and McCormick,¹ who reported on this disease in dizygotic twins. The clinical manifestations and their development will depend on whether the predominate pattern be evidenced by edema or icterus or simply by the blood findings of anemia and increased number of nucleated red blood cells. Hydrops, when associated with either of the other two, is fatal, and the infant is either stillborn or dies immediately after birth. If the jaundice predominates, it may be present at birth or develop shortly thereafter. If present at birth, the vernix may be a golden yellow and the placenta and the amniotic fluid may have an icteric tint. Potter and Adair² point out that neither the anemia nor increased nucleated red blood cells alone are sufficient for a diagnosis of erythroblastosis fetalis and many cases so diagnosed with subsequent recovery are probably erroneous. Buhler et al. record the symptoms in infants as follows: lethargy and exhaustion; symptoms may develop rapidly depending upon the rate of blood destruction; petechial hemorrhages may appear; heart is often enlarged; rapid and weak pulse; no fever usually; liver and spleen are enlarged; and, due to deposition of blood pigment in localized areas of the brain, a syndrome called kernicterus may develop which may quickly cause death. McKinley³ records the early symptoms of kernicterus as convulsions, neck retraction, respiratory paralysis, and drowsiness.

Danis and Anderson⁴ consider the syndromes, congenital hydrops, erythroblastosis fetalis, icterus gravis, and anemia of the newborn, as one condition; namely, a condition known as icterus gravis neonatorum. Their studies on three cases with a favorable outcome and four cases studied by autopsy examinations are quite revealing. Knowing that liver dysfunction was undoubtedly part and parcel of this disease, they studied the faulty fat metabolism in these cases. It was Klemperer⁵ who suggested faulty fat metabolism in this condition, by demonstrating the presence of fatty infiltration with degeneration and necrosis in the cases he studied. The present writers found in the sections of livers of babies dying of this disease fatty infiltration as well as the hemopoietic activity usually found. Griffith⁶ noted a similar fatty change in the liver in choline deficient rats.

¹Buhler, V. B., Seely, C. W., and McCormick, Chas.: *J. Missouri M. A.* 39: 106: 1942.

²Potter, E. L., and Adair, F. L.: *Fetal and Neonatal Death*, Chicago, The University of Chicago Press, 1940, pp. 171-176.

³McKinley, H.: *Arch. Dis. Childhood* 16: 63, 1941.

⁴Danis, P. G., and Anderson, W. A. D.: *South. M. J.* 35: 1070, 1942.

⁵Klemperer, P.: *Am. J. Dis. Child.* 28: 212, 1924.

⁶Griffith, W. H.: *Editorial Reviews* 22: No. 3, Nutrition, Sept. 10, 1941.

It is interesting to note that the prothrombin time in icterus gravis remains high throughout its course on ordinary treatment. This high prothrombin time returned to normal in the three cases that recovered without the complication of kernicterus but this rate of recovery was accelerated by the administration of choline. The **use of choline** came about as follows: the relation of fat metabolism to liver function is uncertain. When neutral fats are absorbed, fat metabolism is carried on in the liver through phospholipid mediation. In the absence of adequate choline, the intermediary catalysis of fatty acids by the liver phospholipid is markedly inhibited. Neutral fat then accumulates in the liver in immense proportions, arising from normal 3 per cent to 27 to 40 per cent. If one feeds cholesterol to rats and guinea pigs a similar accumulation occurs aggravated by choline lack. These writers state that choline is effective through its labile methyl content. It is probable that in the presence of immense fat deposits of triglyceride, the liver cell is functioning under a burden of fat and may deviate from its normal efficiency and metabolism. They therefore added choline to the diet, consisting of Mead Johnson's Olac, thus supplying a form of fat easily absorbable with less possibility of forming cholesterol than butter fat and containing labile methyl through the casein content. This was begun after multiple blood transfusions brought the anemia under control but where the jaundice and liver dysfunction were both at a standstill. In other words, they secured evidence of the rationale of this method of treatment, because they started some time after the effects of the blood transfusions had become manifest and the recovery, which was accelerated, was plainly not due to the transfusions. Obviously, this type of vegetable fat, with labile methyl-containing choline chloride and labile methyl-containing casein, seems indicated in the presence of faulty fat metabolism such as occurs in icterus gravis neonatorum (see pages 1075 ff., exsanguination transfusion).

Laboratory findings,²⁻⁶ are those of a macrocytic hyperchromic, hypochromic anemia associated with a hemolytic type of jaundice. There is an increase in the nucleated red cells. Hemoglobin varies from 25 per cent to 60 per cent. The red cell count is from 1,000,000 to 3,000,000. Reticulocytes are increased from 15 to 20 per cent. Poikilocytosis and anisocytosis are present. Leukocytosis is often marked with an increase in the young white forms. There have been reports of as many as 260,000 white blood cells.* Platelet counts are inconstant and fragility tests variable. Bleeding time is often prolonged; the coagulation time is not affected. The urine contains bilirubin. The stools may be acholic, but more often are not.

Pathologic features are a universal hydrops, with edematous tissues, ascites, hydrothorax, and hydropericardium. Icterus, when present, is the outstanding finding.

*We have not found leukocyte counts of any such proportions. There are many nucleated red blood cells present, the nuclei of which do not dissolve in acetic acid. The white counts should be made under high power, according to the directions on page 577. (R.B.H.G.)

²Bilderbach, J. B., and Bridgeman, M. L.: *Northwest Med.* 39: 85, 1940.

³Diamond, L. K., Cooley, Thomas B., and Josephs, Hugh: *J. Pediat.* 13: 143, 1938.

⁴Diamond, L. K., Blackfan, K. D., and Baty, J. M.: *J. Pediat.* 1: 269, 1932.

⁵Platou, R. V.: *J. Lancet* 61: 151, 1941.

⁶Sanford, H. N.: *Erythroblastosis Fetalis*, *Practice of Pediatrics* (Brennemann), Hagerstown, W. B. Prior Co., vol. 3, pp. 16-22.

Microscopically, the tissues show extramedullary hemopoiesis and blood destruction with islands of erythropoiesis occurring in the liver, spleen, adrenals, kidneys, and occasionally, in other organs. The bone marrow is hyperplastic. Bile deposition and cell destruction are noted in the nuclear areas of the brain. The placental pathology is characteristic, namely, increase in size, grayish or icteric in color with friable, edematous villi. Microscopically the villi are enlarged and the vessels are filled with nucleated red cells.

Those infants exhibiting universal hydrops are either stillborn or die shortly after birth. Those with icterus at birth, or developing icterus within a few hours, carry a worse prognosis than those cases developing jaundice later. In all its forms, the disease shows a high mortality rate.

It is sometimes difficult to make a differential diagnosis between erythroblastosis fetalis and congenital syphilis. Osteochondritis, as shown by roentgenograms of the long bones, may help, also positive serologic findings and history of laboratory evidence of a syphilitic infection in the mother. Hemorrhagic disease of the newborn, accompanied by jaundice, is to be ruled out. Prothrombin determinations, predominate hemorrhagic tendencies, and response to specific therapy help to eliminate this diagnosis.

A case reported by the author¹ is a typical example of this intragroup isoimmunization leading to erythroblastosis fetalis. (See Plates XXVII and XXVIII.)

A pregnant woman was delivered of a baby with erythroblastosis fetalis, which was recognized by the severe icterus and the blood picture. The child lived but a few days, then died. Autopsy showed a very large liver and spleen. **Bone marrow** studies of the baby showed the following picture:

Megakaryocytes	0.25	
Myeloblast	2.50	
Promyelocytes	5.75	
Eosinophilic myelocytes	1.50	
Eosinophilic juveniles	2.00	
Eosinophilic stabs	0.25	
Segmented eosinophiles	1.00	
Neutrophilic myelocytes	13.25	
Neutrophilic juveniles	4.25	
Neutrophilic stabs	0.75	
Segmented neutrophiles	0.75	
Lymphocytes	9.25	
Monocytes	0.25	
Plasma cells	0.50	
Erythrogonia	1.25	
Polychromatic megaloblasts	0.25	} 57.75% nucleated reds
Polychromatic macroblasts	2.25	
Polychromatic normoblasts	54.00	
	100.00	

1 mitotic cell
 Many normoblasts in karyorrhexis
 Most nucleated reds are polychromatic
 Only a very slight reserve of orthochromatic non-nucleated erythrocytes
 Small amount of fat
 Very crowded marrow
 Many free nuclei

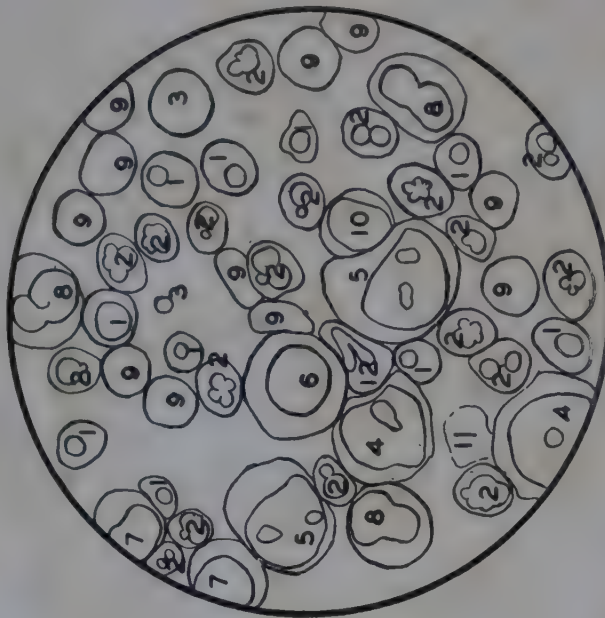
¹Gradwohl, R. B. H.: Lab. Digest 6: No. 2, 1942.

ERYTHROBLASTOSIS FETALIS—FATAL CASE



BLOOD FILM GIEMSA STAIN
X950

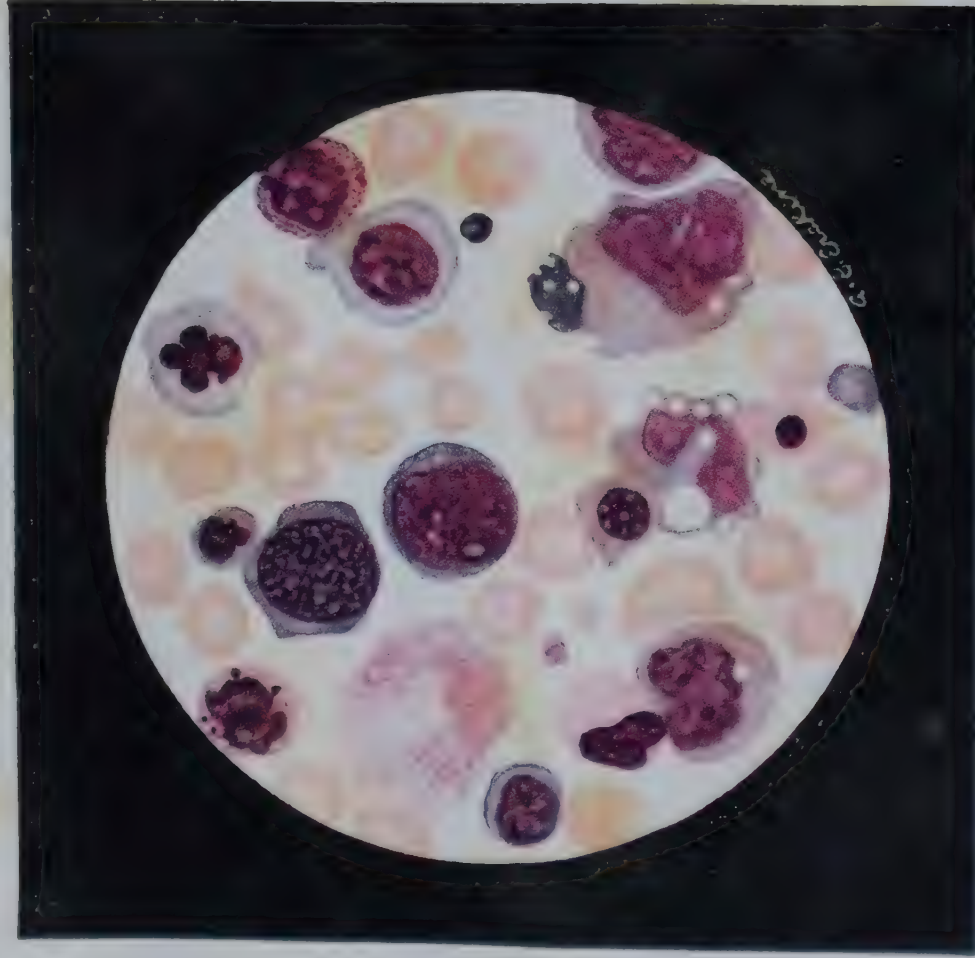
1. Basophile
2. Normoblast in karyorrhexis
3. Macroblood in karyorrhexis
4. Neutrophilic myelocyte
5. Large lymphocyte
6. Small lymphocyte
7. Polychromatic megaloblast
8. Lymphoblast
9. Normoblast
10. Monocyte
11. "Stab"
12. Polychromatic erythrocyte
13. Blood platelet
14. Fragmentary nuclear structure
Note vacuoles in the monocytes



BONE MARROW WRIGHT-GIEMSA STAIN
X950

1. Normoblast
2. Normoblast in karyorrhexis
3. Fat
4. Lymphoblast (?) or myeloblast,
one with Auer rods
5. Promyelocyte
6. Eosinophilic myelocyte
7. Neutrophilic myelocyte
8. Neutrophilic juvenile
9. Lymphocytes (?)
10. Polychromatic macroblast
11. Fragmentary nuclear structure
12. Neutrophilic "stab"

ERYTHROBLASTOSIS FETALIS — FATAL CASE

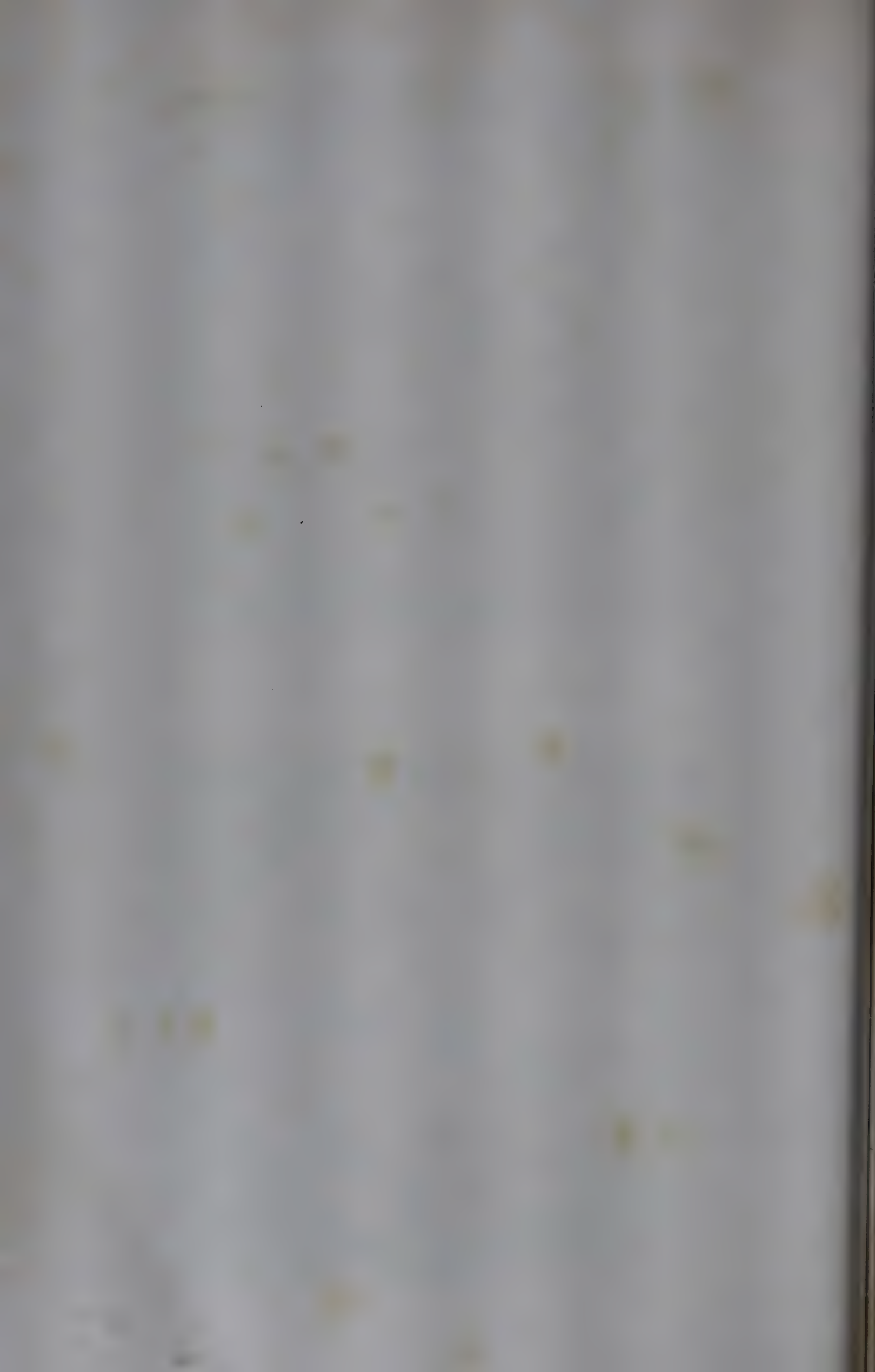


BLOOD FILM
GIEMSA STAIN X950



BONE MARROW
WRIGHT—GIEMSA STAIN X950

PLATE XXVIII.



Complete Blood Count:

Total white count	141,500
Red count	1,950,000
Hemoglobin	49% Sahli
Color index	1.2
Coagulation time	3 minutes
Differential Count:	

Myeloblasts (or lymphoblasts?)	3.0
Promyelocytes	1.25
Basophiles	1.0
Eosinophilic myelocytes	0.25
Eosinophilic juveniles	0.25
Segmented eosinophiles	2.00
Neutrophilic myelocytes	4.25
Neutrophilic juveniles	7.00
Neutrophilic stabs	16.25
Segmented neutrophiles	10.75
Lymphocytes	36.75
Monocytes	16.00
Plasma cells	1.25
	<hr/>
	100.00

For every 100 leukocytes, there were

20 Polychromatic normoblasts
 3 Polychromatic macroblasts
 5 Polychromatic megaloblasts
 0.25 Erythrogonia.

Many cells highly vacuolated and atypical.

Toxic granules of neutrophiles.

Many fragmentary nuclear structures.

2 Giant segmented neutrophiles.

Macrocytes present.

Anisocytosis.

Marked, intensely blue, polychromasia.

Karyogenic metachromasia.

The father's blood was Rh positive: the baby's blood was Rh positive: the mother's blood was Rh negative. The mother's blood when tested failed to show anti-Rh agglutinins in the blood serum. This was the second pregnancy in this patient that resulted in erythroblastosis fetalis: first two years prior to this recent event. It is interesting to note that her first pregnancy, six years ago, resulted in a healthy child. This observation has been made by Levine; namely, that isoimmunization to the extent of always producing hemolytic changes or icterus gravis in the fetus does not occur with every pregnancy, but that usually the first child is spared.

Agglutinins were not found in the mother in this case because it goes back to 1942. Since then improved technics, involving tests for univalent or blocking antibodies, would reveal such agglutinins in the mother.

Congenital Leukemia

A number of cases of congenital leukemia have been noted in the literature. They are usually of the acute type; they may be myelocytic or lymphocytic.

Congenital Malaria

Congenital malaria is looked for and expected in malarial districts. The diagnosis is made by finding the characteristic blood picture of malaria including plasmodia.

Congenital Syphilis

In this disease, there is no characteristic blood picture. We see a very severe anemia, hemorrhagic diathesis, and blood serology, which are sufficient to make the diagnosis.

Congenital Anemia

A number of cases of so-called congenital anemia have been reported. These show anisocytosis, poikilocytosis, and polychromasia.

It is claimed that there are two primary anemias. The etiology is based upon a toxic irritation or a primary weakness in the hematopoietic apparatus. It is interesting to note that anemia of the mother is usually not carried over to the child, not even in severe cases of pernicious anemia. This applies both to the pernicious-like anemia, which has been called a pernicious-pregnancy-anemia, and true pernicious anemia. It is also true that maternal leukemia is not carried over to the child.

Anemias of Infancy and Childhood

Anemia is very common in these young subjects. Attention has already been called to the discrepancies in the figures of hemoglobin and red cells between infancy and adult ages. The physiologic anemia of premature babies has already been considered. A classification of anemias in children is more difficult than it is in adults. The following groups may be considered:

1. Anemia perniciosa of Biermer.
2. The "aplastic anemia" of Ehrlich, also called "hemolytic anemia" of Türk, or the "aregenerative anemia" of Pappenheim, or the "aleukia hemorrhagica" of Frank.
3. Progressive postinfectious anemia.
4. Congenital and inherited hemolytic icterus.

Whether true pernicious anemia occurs in children is debatable. Most of the cases that have been described with pernicious anemic blood pictures are connected with *Diphyllobothrium* infestation.

Anemia Pseudoleukemica Infantum of von Jaksch

This is a special disease of children between the ages of six months and two years. It is an anemia of moderate degree with a high-grade dystrophy and enlargement of the spleen. Most of these children are rachitic. The following hematologic changes are seen:

1. Definite decrease in cell volume.
2. Definite decrease in hemoglobin content.
3. Marked decline in number of red cells.
4. High-grade progressive increase in leukocytes.
5. Changes in size and morphology of the red cells together with the presence of many nucleated red cells. There are many karyokinetic figures.
6. Relative quantities of white cells to red cells, as in the proportion of 1:100.

Baar and Stransky¹ describe a typical case as follows:

E. N., six months old, twin, normal birth, weighed 1,600 gm. at birth. The twin sister weighed 1,850 gm. Breast fed for two months, then fed mother's and cow's milk, equal parts. For four weeks had daily 800 or 900 gm.—2/3 milk and vegetables twice daily.

¹Baar, H., and Stransky, E.: *Klinische Hämatologie des Kindesalters*, Franz Deuticke, Leipzig und Wien, 1928, p. 86.

Patient affected with grippe for last fourteen days, with fever four days at 39.5° C.; since then fever-free. Patient since this time showed great pallor and poor appetite. Stool normal. Mother has a bronchial catarrh; father healthy. Twin sister healthy, not pale. Status praesens: Small, weak, poorly nourished, pale child. Skin high-grade anemic with a yellow color tone. Visible mucosae pale. Lobes of the ears by transmitted light almost colorless. Few ecchymoses of the skin. Definite craniotabes rachitica, rachitic rosary. Thorax weak. No lymph gland enlargement. Chvostek negative. Throat mucosa pale. Lung findings negative. Heart findings negative. Abdomen distended. Spleen reaches to the umbilicus. Splenic tumor extends four fingerbreadths below the left costal border. Liver four fingerbreadths below the right costal border. Urine shows increased urobilinogen. Blood findings: hemoglobin (Sahli) 30 per cent; erythrocytes 2,060,000; color index 0.75; leukocytes 24,100; blood platelets 40,000; stabs 1.8; segments 21; lymphocytes 72.4; monocytes 2.8; eosinophiles 1.2; basophiles 0.2; neutrophilic myelocytes 0.2; myeloblasts 0.2; plasma cells 0.2. Red cells show anisocytosis, poikilocytosis, and polychromasia.

In general, anemia infantum pseudoleukemica is a symptom complex with anemia, erythroblastosis, myelogenous reaction, and a splenic tumor.

Erythroblastic Anemia or Cooley's Anemia

Under this title Cooley and Lee² reported five cases which showed symptoms similar to the von Jaksch disease just described. Cooley, Witwer, and Lee³ later reported two more similar cases. All these cases showed severe secondary anemia, enlarged spleens, leukocytosis of varying degrees, many normoblasts, and normal or increased fragility to salt solutions. Others have reported similar cases.⁴ In this connection, Cooley believes that von Jaksch probably erred in stating that many of these cases recovered, due no doubt to the fact that in the group originally classified by von Jaksch, there were many cases of ordinary secondary anemia which did not properly belong to this group. At the same time, von Jaksch, in his original paper in 1889, did not emphasize the occurrence of nucleated reds nor the appearance of immature white cells in these cases.

For information concerning the disease, now spoken of as "thalassemia," and for the blood picture, refer to page 731.

Chlorosis

The question has been raised—does a child ever have a true chlorosis, or is it a chlorotic-like disease that we see in these young subjects? The definition of chlorosis, as given by von Noorden follows:

1. A disease of female puberty with tendency to recurrence.
2. Cardinal symptom of this disease is anemia, decline in hemoglobin, and number of red cells. As a result of the anemia, we have the various symptoms of the disease.
3. The disease develops spontaneously without apparently any existing cause.

²Cooley, T. B., and Lee, Pearl: Tr. Amer. Pediat. Soc., 1925.

³Cooley, Witwer, and Lee: Am. J. Dis. Child. 34: 347, 1927.

⁴Hitzrot: Ann. Surg. 88: 361, 1928.

Whipple, Reeves and Cobb: Ann. Surg. 88: 380, 1928.

Baty, Blackfan, and Diamond: Am. J. Dis. Child. 43: 667, 1932.

Crawford and Williamson: Am. J. Dis. Child. 46: 565, 1933.

4. The disease is only a disturbance of the nutritional state of the blood without any disturbances in the general nutritional state of the individual affected.

It is generally conceded that true chlorosis does not occur in children, but that we have at times a chlorotic-like anemia. Baar has described a pseudochlorosis infantum characterized by lack of iron in the blood stream.

Anemia of Scurvy

In scurvy, we find a characteristic bone marrow disturbance which was well described by Senator. Cases of scurvy anemia have been fully described in the literature.

There is an anemia which is seen in cases of dystrophia avitaminosa. These cases have been recorded in connection with so-called "goat-milk dystrophia" and "goat-milk anemia."

Alimentary Anemias

Alimentary anemias in pediatrics may be divided into the two following groups:

1. Pseudochlorosis infantum or infantile lack of iron, or infantile iron-poor anemia.
2. Alimentary infectious anemia.

Infectious and Postinfectious Anemia

These cases follow such infections as congenital syphilis, sepsis, and malaria. In tropical countries, it is very often difficult to distinguish between chronic malaria in children and leishmaniasis infantum. In chronic malaria, there is an early appearance of fever, gastrointestinal disturbance, spleen and liver edema, and anemia with pallor. In leishmaniasis there is a late development of these symptoms, followed by a late fever and gastrointestinal symptoms. In chronic malaria, there is swelling of the liver and spleen with a sensitiveness of these organs particularly to pressure in the neighborhood of the phrenic nerve. In leishmaniasis, there is swelling of the liver and spleen but no sensitiveness. In chronic malaria, there are gastrointestinal disturbances more often with constipation than diarrhea, whereas in leishmaniasis, gastrointestinal symptoms occur but more often with diarrhea than constipation. The edema in chronic malaria has certain definite localizations, such as the face or the lower extremities, whereas in leishmaniasis, the edema is seldom in the face and more often on the lower extremities. In leishmaniasis the blood shows an oligemia; erythroblasts are rare. White cells are decreased. There is usually a relative lymphocytosis. Resistance of the red cells to hemolysis in hypotonic salt solution is lowered. Parasites may be found in the blood very rarely but can be found in splenic puncture.

Hematoxic Anemia

Anemias due to poisons such as lead, potassium chlorate, pyrogallol, etc., are not different in children than they are in adults. There are many cases

of lead anemia seen in infants and children. It is interesting to note that a symptom of lead poisoning in infants is a convulsion.

Lead anemia has already been described. (See Hematology of Lead Poisoning.) A very important symptom of lead poisoning in children is the deposit of lead in the ends of the growing bones which can easily be determined by roentgenologic examinations.

Anemia Due to Intestinal Parasites

Severe anemias in children due to intestinal parasites are very common. These are seen in connection with infestation with *Diphyllobotrium latum* and *Taenia*. Theodor, Cima, Naegeli, and others have repeatedly called attention to anemias due to infestations due to *Trichuris trichiura*. Cima, Cozzolino, Ashford and others have described anemias due to *Ancylostoma duodenale*; and those due to *Schistosoma haematobium* have been described by Kautzky-Bey. Stäubli, van Cott, and others have described the anemias due to trichinosis. Kautzky-Bey claims that *Schistosoma haematobium* and not *Ancylostoma duodenale* is the causative agent of the so-called "Egyptian anemia" in children. "Egyptian anemia" is characterized by a disturbed relationship between the hemoglobin content and the number of red cells and by the presence of oligochromemia and a marked eosinophilia. In trichinosis in children there is at first a polycythemia and later a slight hypochromic anemia. *Ascaris* and *Enterobius* (*Oxyuris*) infestations do not often produce anemizing symptoms.

Constitutional Anemia

By "constitutional anemias" is meant those due to some cause other than poisoning or infections. Many of these cases are due to disturbances of the glands of internal secretion. Some are due to a let-down in the physiologic activity of the hematopoietic organs. In this latter type are seen anemias in connection with stigmata of degeneration and hydrocephalic children.

The blood picture seen in these cases is anemia without any indications of a regenerative tendency. To these constitutional anemias belong such diseases as erythrodermia desquamativa and also sickle cell anemia. The anemia connected with internal glandular secretory function is seen particularly in myxedema. Here is seen an oligocythemia, oligochromemia, relative lymphocytosis, leukopenia, and often an eosinophilia. "Kocher" blood picture is also characteristic in myxedema; namely, lymphocytosis and neutropenia.

Pernicious Anemia

True pernicious anemia is seldom if ever seen in children. Most cases are really "pernicious anemia blood pictures" due to infestations by worms. It is generally conceded that children are not disposed toward pernicious anemia.

Aplastic Anemia

Aplastic anemia was first described by Ehrlich in 1888. It has also been called hemorrhagic aleukia, panmyelophthisis of Frank, and aregenerative asthenic anemia of Pappenheim. For fuller details, see remarks under aplastic anemia in adults. Eppinger has divided this into two groups—acute and

chronic aplastic anemia. Typical cases of aplastic anemia are very rare in children. Most cases of so-called aplastic anemia in children are really cases of aleukemic leukemia with aplastic blood pictures.

Pseudoanemia (Sahli)

This has already been described. See page 765.

Acute Leukemia of Children

According to Fraenkel, this is a form of leukemia which begins suddenly and has a rapid termination. The first case reported was by Friedreich¹ in 1857. Up to 1889 Epstein had reported sixteen cases. Of the forty-five cases collected by Theodor² in 1897, nine occurred in children. A monograph of Benjamin and Sluka³ in 1907 reported thirty-six cases of acute leukemia in children from the age of 2 to 14 years.

These cases in children begin with fever, headache, pains in the legs, weakness, vomiting, and chills. They show a triad of symptoms, anemia, hemorrhagic diathesis, and ulcerative, gangrenous changes in the digestive tract, especially in the mouth. Other workers have described nodular changes in the skin as part of the acute leukemic process. Hemorrhagic diathesis, seen in the form of hemorrhages under the skin and visible mucosae and sometimes rectal hemorrhages, has been reported. Hemorrhage in the cerebrum may lead to hemiplegia. Subperiosteal hemorrhages occur. Swelling around the tibia has been reported. Changes in the central nervous system are seen in the form of polyneuritis.

The lymph glands are enlarged. Spleen is enlarged. The heart is enlarged; there is a small, accelerated pulse. Sometimes hemorrhagic nephritic symptoms are seen. Septic symptoms are noteworthy.

Blood picture in acute leukemia of children shows changes not only in the white cells but also in the erythrocytes and blood platelets. There is a severe progressive anemia combined with a subicterus. The red cell count may go below 1,000,000. The hemoglobin does not keep pace with the drop in red count, so that the color index is high. Megalocytes and megaloblasts may be seen, so that these cases have sometimes been mistaken for pernicious anemia. The number of white cells is so increased that the relativity of white cells to red cells is about 1:100. Wollstein and Bartlett⁴ refer to the aleukic stage of acute leukemia, where there is marked change in the differential picture, but not a great increase in the total number of white cells. In acute leukemia in children, we see large, nongranulated, single-nucleated cells. These juvenile lymphocytes in the case of acute lymphocytic leukemia are so definite that we can make a diagnosis upon their appearance. In the acute myelocytic leukemias, we see many mononucleated cells, the myelocytes of Ehrlich. There are also mononucleated eosinophilic leukocytes. There are many mitoses seen in the white cells. Resort must be had to the Graham oxydase stain to distinguish myeloid from lymphatic cells.

¹Friedreich, W.: *Virehows Arch.* 12: 37, 1857.

²Theodor: *Arch. f. Kinderh.* 22: 47, 1897.

³Benjamin, E., and Sluka, E.: *Jahrb. f. Kinderh.* 65: 253, 1907.

⁴Wollstein, M., and Bartlett, F.: *Am. J. Med. Sc.* 169: 819, 1925.

Chronic Leukemias of Children

Chronic leukemias of children are very common. They begin very insidiously with weakness, loss of appetite, and general disturbances in the nutritional status. The spleen enlarges slowly. The lymphatic glands enlarge later. Blood picture is very characteristic. Hemoglobin content and the number of red cells may be normal in the beginning but later declines. Later we see signs of chronic anemia, polychromasia, anisocytosis, poikilocytosis, and presence of normoblasts and megaloblasts.

At times, it is necessary to make a differential diagnosis between the blood picture of an infectious leukocytosis and that of a chronic leukemia in children. In both conditions the count of the leukocytes is increased, but eosinophiles and monocytes are low or absent in septic leukocytosis but are present in large amounts in chronic myelocytic leukemia.

Chronic lymphocytic leukemia in children has been described by a number of writers. A typical case of a 13-year-old boy is as follows:

Weakness, loss of appetite. Blood picture, 32 per cent hemoglobin, 1,570,000 red cells, and 463,000 white cells. In the film, 98 per cent lymphocytes, no myelocytes, very little anisocytosis or poikilocytosis, occasional normoblasts. Blood platelets are scarce.

Leukemias of Nurslings

Leukemias of nurslings are very rare. From the time of the discovery of this disease in 1845, by Virchow, up to 1914, only seventeen cases had been reported. Since then many have been reported.

Symptomatic Megalosplenism or Banti's Disease

The term "splenic anemia" does not refer to any specific disease; it is simply a term used to describe those cases with both enlargement of the spleen and anemia.

Splenic enlargement may occur in the following conditions: (1) in anemias—either a secondary regenerative form or in hemorrhagic anemia; (2) in the hemorrhagic diatheses, there is megalosplenism; (3) polycythemia; (4) in lymphatic leukemia or lymphogranuloma; (5) in myelocytic leukemia or in aleukic myeloses; (6) chronic infections, such as congenital syphilis, malaria, leishmaniasis, etc.; (7) in rickets in first year of life—later on, it disappears; (8) in acute infections, as typhoid fever, miliary tuberculosis, and septic infections; (9) Gaucher's disease and Niemann-Pick disease; (10) in localized splenic tuberculosis; (11) in Banti's disease where there is a fibroadenoma of the spleen, liver cirrhosis, and finally, anemia; (12) splenic tumors from circulatory disturbances such as cardiac stagnation in beginning heart failure, or beginning heart failure from valvular disease, or stagnation from acute or chronic thrombosis in the venae cavae, or through stagnation by reason of pericardial adhesions; (13) splenic tumor in liver cirrhosis.

Banti's disease is accompanied by enlargement of the spleen without any change in the lymphatic glands, followed by symptoms of simple anemia and by weakness, dyspnea, etc. Hemoglobin content may drop to 30 per cent. There follows a second period during which the liver enlarges without icterus and without ascites, but the splenomegalia remains. This lasts perhaps from

eight to thirteen months. The third period is accompanied by ascites and decrease in size of the liver. There is an atrophic cirrhosis in the liver. It occurs after hemorrhages or through the progressive liver atrophy. The number of red cells is not markedly decreased, but the color index is lower. Nucleated red cells are not seen. The lymphocytes are usually decreased. Histologic investigations of the organs in fatal cases of Banti's disease show a fibro-adenosis of the spleen, including the pulp as well as the follicles. In the second stage of the disease, there is a sclerotic condition of the intrahepatic veins, later a hypertrophy of the interstitial material. No siderosis is seen in the liver.

Blood Picture.—There is a marked and constant leukopenia, sometimes the white cells are down to 1,000. There is no change in the differential picture. As already noted, the anemia progresses with the advance in the disease. There is a low color index. There may be as few as 3,000,000 red cells with 40 per cent hemoglobin. Hemorrhages into the stomach are followed by further reduction in the red cells and hemoglobin percentage, together with the temporary posthemorrhagic leukocytosis. The anemia itself may be severe enough to be fatal. There is an increased resistance of the red cells.

Hemorrhagic Diseases of Children

Basically the mechanisms involved in hemorrhagic diseases in children are similar to those in hemorrhagic diseases in adults.

NORMAL BLOOD FINDINGS IN DOMESTICATED ANIMALS

The use of various animals for laboratory purposes requires some knowledge of the normal blood picture of these laboratory and domesticated animals.

Guinea Pigs.—

Guinea pigs have an average number of red cells of 5,250,000. The hemoglobin varies from 85 to 100 with an average of 94. The total number of leukocytes varies from 5,000 to 22,000 with an average of 11,000 to 12,000. Guinea pigs have an average of 350,000 blood platelets per cubic millimeter.

Rabbits.—

In rabbits, the number of red cells varies from 5 to 8 millions with an average of 6 millions. Average hemoglobin value is 96. The number of leukocytes ranges from 8,000 to 13,000 with an average of 11,000. The differential picture is lymphocytes, 45-55; monocytes, 2-8; polymorphonuclears, 40-50; eosinophiles, 0.5-1 and basophiles, 4-8. We have noted an average reticulocyte count of 4 to 6 per cent.

Cats.—

Total number of red cells is about 6 millions. Goodall¹ found the normal to vary from 7.28 to 8.6 millions with an average of 8 millions. There are 7,000 to 19,000 leukocytes with an average of 13,300. Goodall found higher numbers of leukocytes. The differential picture is monocytes, 37; neutrophils, 54; eosinophiles, 9; and mast cells (basophiles), 1.0.

¹Goodall, A.: J. Path. & Bact. 14: 195, 1910.

Dogs.—

Red cells range from 5 to 8 millions with an average of about 6 millions. Hemoglobin values vary from 55 to 104, with an average of 90. Different investigators have reported different quantities of leukocytes in dogs. Six thousand to 12,000 with an average of about 8,000 seems to be the normal for adult dogs. The differential picture is lymphocytes, 11-29, average 20; monocytes, 3-10, average 6; neutrophiles, 60-76, average 68; eosinophiles, 1-10.5, average 6; with basophiles very rarely found.

Goats.—

Red cells are 9 to 10 millions per cubic millimeter in amount. Leukocytes are around 10,000 per cubic millimeter.

Sheep.—

Average number of red cells is 8 millions and of leukocytes is 8,000. Amount of hemoglobin varies from 47 to 63 per cent. The percentage of white cells in the differential picture in sheep is lymphocytes, 40 to 60; monocytes, 3 to 11; neutrophiles, 30-55; eosinophiles, 0.2 to 0.8; basophiles, 0 to 2.

Cows.—

Red cells vary from 5 to 8 millions, with an average hemoglobin range of 45 to 85. Normal number of leukocytes is 5,000 to 10,000. Differential picture, according to Dimock and Thompson,² is lymphocytes, 54.2%; monocytes, 1.4; neutrophiles, 30.5; eosinophiles, 13.15; basophiles 0.59.

Horses.—

Normal number of red cells for stallions is from 7 to 10 millions, with an average of 8 millions; for geldings 5½ to 9 millions with an average of 7½ to 8 millions; for mares, 5½ to 7½ millions with an average of 6½ to 7 millions. The hemoglobin varies from 85 to 100. Leukocytes vary from 5,000 to 10,000, with an average of 8,000. The differential picture is lymphocytes, 34 to 38; neutrophiles, 60 to 65; eosinophiles, 1 to 2.

Swine.—

Normal count of red cells is about 8 millions, slightly less for young pigs. Hemoglobin values are around 85. Leukocyte counts are 19,000 with more than 50 per cent of them lymphocytes. The differential picture of swine shows lymphocytes, 56.4; neutrophiles, 38; eosinophiles 5.13.

²Dimock and Thompson: *Am. Vet. Rev.* 30: 553, 1906.

CHAPTER V

BLOOD GROUPS AND TRANSFUSION*

The first record of a blood transfusion is referred to in classical literature: Medea attempted to rejuvenate old Anchises by removing blood from his cervical vessels and replacing it with juvenile blood (Ovid). Obviously no genuine blood transfusion could have been performed prior to the discovery of the circulation of blood. Thomas Clark and Henshaw, soon after the discovery of the circulation of blood by William Harvey in 1616, began to make animal experiments on blood transfusion. Denys and Emmerez of Paris in 1667 made a direct transfusion of the blood from the carotid artery of a sheep into the cubital vein of a human patient, and good results followed. Others repeated this, but disastrous results occurred in some cases, so that it was abandoned. During the past century, blood transfusion was revived.

Owing to the fact, however, that severe reactions and death followed the transfusion of incompatible bloods, the procedure was practically abandoned for many years. It was revived only when methods were discovered for determination of the compatibility of human bloods. At the present time, methods of blood grouping and blood matching have eliminated many of the dangers incident to transfusion. It is now known that in most instances the unfavorable reactions were due either to hemolysis or to agglutination of either the donor's or recipient's blood cells or to both.

BLOOD GROUPS

Landsteiner¹ established the basis for present methods for determination of compatibility or incompatibility of bloods for transfusion. He discovered that all human beings can be divided into three distinct groups. He showed that the identity of each group depended upon the presence in the red blood cells of isoagglutinogens. He called these groups A, B, and C (not O). Group C was later called "O." A fourth group was discovered by von Decastello and Sturli.² This was the AB group. It was found that in the case of the group A, the serum reacted upon the corpuscles of group B, causing agglutination, but the serum of group A did not react upon the corpuscles of group A. The corpuscles of group A, in turn, were agglutinated or reacted upon by the serum of group B, but the serum of group B did not react upon the corpuscles of group B. It is obvious, therefore, that normally the corpuscles are apparently not sensitive to their own sera.

*We are indebted to Dr. Lester J. Unger, Director of the Blood and Plasma Bank, New York University-Bellevue Medical Center, New York City, N. Y., for his kind assistance in the revision of this chapter. Besides giving us the benefit of his advice and suggestions, Dr. Unger permitted my assistant, Mrs. Addine G. Erskine, to work in his laboratories to obtain first-hand knowledge of his technics so that they could be given here. It is our belief that this chapter, as now rewritten with the assistance of Dr. Unger, is the most complete treatise on this subject yet to appear in any textbook.

We also wish to thank Dr. Alexander S. Wiener, Brooklyn, N. Y., for having kindly read the entire manuscript.

¹Landsteiner, K.: *Wien. klin. Wchnschr.* 14: 1132, 1901.

²Münch. med. Wchnschr. 49: 1090, 1902.

There are four A-B-O blood groups, designated O, A, B, and AB, according to the International Classification. Their approximate percentages among representative American and European peoples are: O, 45 per cent; A, 42 per cent; B, 10 per cent; AB, 3 per cent.

There were two other classifications, Moss¹ and Jansky,² which are no longer used, but merely as a matter of record their equivalents according to the International Classification are given in Table 95.

The **International Nomenclature**, officially recognized by the Health Committee of the League of Nations, has been adopted. It is the same as the original classification of Landsteiner and von Dungern and Hirszfeld and is the nomenclature of choice; namely, O, A, B, and AB. To avoid confusion, it is urged that the group names of Moss and Jansky be eliminated from medical literature.

A further interesting communication upon blood groups was that by von Dungern and Hirszfeld,³ who discovered the existence of subdivisions in two of the four human blood groups.

It is interesting to note that the substances which characterize the four blood groups are present in practically every tissue of the body and have been found in soluble form in the serum, saliva, gastric juice, semen, urine, etc.

In addition to the foregoing facts regarding the primary four groups, Landsteiner and Levine⁴ noted that when certain immune sera from rabbits, previously injected with human blood, were absorbed with certain samples of human blood, they still contained agglutinins acting on the majority of bloods of all four groups, while other bloods were not agglutinated. Two of the factors demonstrable by these sera were designated **M** and **N**. They found that either the M or N agglutinogens or both were present in all human blood cells but no anti-M and anti-N agglutinins were found in the sera. These may be produced by injection of rabbits with the appropriate type of cells. Research since that time has shown that all human bloods may be divided into three types, M, N, and MN. The agglutinogens M and N are hereditary just as are the other blood groups originally described by Landsteiner. The **M** and **N** factors are of almost no importance with respect to blood transfusion work, for the reason that there are hardly ever any anti-M and anti-N agglutinins in normal human blood sera and M and N agglutinogens are apparently only feebly antigenic for human beings. They are important, however, in questions of determination of paternity, but are unreliable in the identification of blood stains. These facts are described on pages 1168 ff. and 1172 ff.

At What Age Do Agglutinogens and Agglutinins Appear in the Blood?—

Kemp⁵ showed that the isoagglutinin could first be demonstrated in the red blood cells of a fetus, 37 days old, and demonstrated that the sensitivity of the cells increases throughout antenatal and postnatal life up to the age of

¹Moss, W. L.: Bull. Johns Hopkins Hosp. 21: 63, 1910.

²Jansky, J.: Sborn, lin. 8: 85, 1906-07.

³Ztschr. f. Immunitäts. 8: 526, 1911.

⁴Landsteiner and Levine: Proc. Soc. Exper. Biol. & Med. 24: 600, 941, 1927; J. Exper. Med. 47: 757, 1928.

⁵Acta pathol. et microbiol. scandinav. 7: 146, 1930.

20 years after which time it remains constant.¹ He also noted that the red blood cells of the newborn infant generally have only 20 per cent of the sensitivity to agglutination that adult blood cells have. Expressed in other terms, a serum with a titer of 500 against adult corpuscles would usually agglutinate the corpuscles of the newborn infant only up to a dilution of 1 to 100. Thompson and Kettel² determined the titer of the isoagglutinins in the sera of individuals of various ages. They found that isoagglutinins begin to appear in the latter half of antenatal life and rapidly increase in titer after birth up to the age of puberty. At this time, the titer gradually diminishes. It is generally considered that the average titer of the α (anti-A) isoagglutinin is higher than that of the β (anti-B) isoagglutinin. Wiener states that while isoagglutinogens are demonstrable at birth, only about half of all newborn infants have demonstrable isoagglutinins. Hirszfeld³ believes that any isoagglutinins at birth are derived from the mother by filtration through the placenta. Smith⁴ studied the isoagglutinin and isoagglutinin content of the blood of infants from day to day during the first few weeks of postnatal life and found that whatever isoagglutinins were present at birth diminished in titer or disappeared during the first ten days of life, after which time new isoagglutinins appeared.

Bornstein and Israel^{5, 8} made a study of agglutinogens in fetal erythrocytes, particularly the M and N agglutinogens. Moureau⁶ demonstrated the factors **M** and **N** in very young fetuses. Hyman⁷ found M and N demonstrable in three fetuses but failed to obtain either M or N in the fourth, 3 months old, whose cells exhibited the isoagglutinin B. She left the question open whether agglutinogens had disintegrated before expulsion of the fetus or whether they had not yet developed at that time.⁸ Bornstein and Israel's study comprised a larger series of fetuses to determine whether or not their agglutinogens are detectable with sera and testing fluids used in transfusion work and in legal tests. In addition, by including the Rh factor in the investigation, information can be obtained on the value of tests performed with fetal blood for investigations on isoimmunization as a cause of habitual abortion, according to Levine.⁹

Their series comprised nineteen premature fetuses, only those showing gross evidence of maceration being omitted. Blood was taken from the heart, suspended in physiologic saline solution, and examined the same day with the centrifuge method. In some cases examination of parents was possible. Various human A and B antisera and M and N testing fluids were used, which gave positive agglutinations with the corresponding cells in dilution of 1:8. For the Rh tests the test tubes were incubated for one hour at 37° C. before centrifugation.

¹Quoted from Wiener, A. S.: *Blood Groups and Blood Transfusion*, Springfield, Ill., Charles C Thomas, p. 16.

²*Ztschr. f. Immunitäts.* 63: 67, 1929.

³See Hirszfeld, L.: *Konstitutionsserologie und Blutgruppenforschung*, Berlin, 1928, Julius Springer. Also see: Hirszfeld and Zborowsky: *Klin. Wchnschr.* 4: 1152, 1925.

⁴*Am. J. Dis. Child.* 36: 54, 1928.

⁵Bornstein, S., and Israel, M.: *Proc. Soc. Exper. Biol. & Med.* 49: 719, 1942.

⁶Moureau, P.: *Rev. belge sc. méd.* 7: 540, 1935.

⁷Hyman, H. S.: *Abstr. Doct. Diss.*, No. 21, The Ohio State University Press, 1937.

⁸Hyman-Parker, H. S.: *J. Immunol.* 43: 1, 1942.

⁹Levine, P., Burnham, L., Katzin, E. M., and Vogel, P.: *Am. J. Obst. & Gynec.* 42: 925, 1941.

The fetuses varied in length between 7 and 50 cm., the average being 28.6 cm. The distribution of agglutinogens was independent of the length of the fetuses and did not grossly deviate from the percentages in the population. No fetus without either M or N was encountered, and no combination parent M, fetus N, or vice versa occurred. The Rh factor was demonstrable in four out of five fetuses. This confirms the indirect evidence that the Rh factor is present early in fetal life as derived from Levine's work.

Table 93 gives the summary of their work :

TABLE 93.—BLOOD GROUPS AND BLOOD TYPES OF FETUSES AND PARENTS. PRESENCE OR ABSENCE OF RH FACTOR (TESTED IN NOS. 15-19 ONLY)

FETUS				
NO.	LENGTH	AGGLUTINOGENS	MOTHER	FATHER
1	21 cm.	O MN	O M	
2	7 cm.	O MN	B M	
3	13 cm.	A M	A M	
4	47 cm.	O MN		
5	23 cm.	A M		
6	13 cm.	O M	O MN	O MN
7	30 cm.	A MN	A MN	A MN
8	50 cm.	A M		
9	42 cm.	A N		
10	27 cm.	A MN		
11	19 cm.	O N		
12	26 cm.	B M		
13	30 cm.	A N		
14	33 cm.	B N	A MN	
15	41 cm.	AB M Rh+	A MN Rh+	
16	42 cm.	A M Rh+	O MN Rh+	
17	39 cm.	A MN Rh-	A MN Rh-	
18	17 cm.	O N Rh+	A N Rh+	
19	21 cm.	B MN Rh+	A MN Rh-	B N Rh+

This research demonstrated agglutination of A, B, M, N, and Rh in the heart's blood of nineteen fetuses. This seems to prove that agglutinogens are actually developed long before birth and in sufficient strength to make the examinations reliable enough for scientific purposes.

Composition of the A-B-O Blood Group System

Division of all bloods into four groups of the A-B-O system depends upon antibodies contained in the plasma and blood factors found in the cells. The substance in the cells is called *isoagglutinogen*, while the antibody in the plasma is called *isoagglutinin*. Now, at least 10 different blood group systems have been described; namely, the A-B-O, M-N-S-s, P, Rh-Hr, Lewis, Lutheran, Kell-Cellano, Duffy, Kidd, and U* blood group systems. For each system, one or more characteristics or factors have been identified.

The A-B-O group name is derived from the isoagglutinogen content of its cells. The isoagglutinins are designated as anti-A and anti-B, or by the Greek letters α and β , while the isoagglutinogens are designated by the Latin letters A and B. Isoagglutination, or the clumping of human blood cells by the action of an incompatible human serum, occurs when the specific isoagglutinin and isoagglutinogen are brought into contact with each other. Since anti-A is specific in its action on A isoagglutinogen, the sera of all bloods containing

*Wiener and Unger: J. A. M. A. 153: 1444, 1953.

anti-A will agglutinate the cells of all bloods containing the **A** factor. Similarly, the anti-B agglutinin of blood serum will agglutinate all blood cells containing the **B** factor. The names for most of the other systems were derived from the individuals in whom the antibody was first found.

There are two isoagglutinins, anti-A and anti-B, and two isoagglutinogens, A and B. Group O contains in its plasma both isoagglutinins anti-A and anti-B, while the red cells contain the isoagglutinogen O (or none). The group is identified by reason of the fact that its corpuscles are insensitive to the isoagglutinins, whereas its serum will agglutinate the red cells of all other groups. Group A contains in its plasma the isoagglutinin anti-B, while its erythrocytes contain the agglutinable substance A. Thus the plasma of group A will agglutinate cells of all groups containing the agglutinable substance B (groups B and AB), while its cells are agglutinated by all groups whose sera contain the anti-A isoagglutinin (groups O and B). Group B contains the isoagglutinin anti-A in its plasma, while the isoagglutinogen B is found in its cells. Thus the serum of group B will agglutinate all cells containing the A agglutinable factor (groups A and AB), while its cells will be agglutinated by all sera containing the anti-B isoagglutinin (groups O and A). Group AB contains no isoagglutinins in its serum, but its cells contain both the A and B isoagglutinogens. Its red cells are therefore agglutinated by sera of all other groups, while its serum has no action upon the red cells of any groups. These facts are seen in Table 94.

TABLE 94

		REACTION ON RED BLOOD CORPUSCLES OF GROUPS			
		O	A	B	AB
SERUM OF GROUPS:	O	-	+	+	+
	A	-	-	+	+
	B	-	+	-	+
	AB	-	-	-	-

-, not agglutinated.
+, agglutinated.

Table 95 represents the groups under the three nomenclatures, isoagglutinin and isoagglutinogen content, and the per cent of the population in each group.

TABLE 95

INTERNATIONAL, OR LANDSTEINER, OR V. DUNGERN AND HIRSZFELD	JANSKY	MOSS	ISOAGGLU- TININS IN SERUM	ISOAGGLUTINO- GENS IN CELLS	APPROXIMATE PER CENT OF AMERICANS (CAUCASOIDS) IN EACH GROUP
O	I	IV	Anti-A and Anti-B (α and β)	O	45
A	II	II	Anti-B (β)	A	42
B	III	III	Anti-A (α)	B	10
AB	IV	I	None	A and B	3

In order to insure safe blood transfusions,* that is, in order to prevent what is known as incompatible reactions, certain preliminary examinations must be made of both the recipient's and the donor's blood. These are as follows: (1) Obtain the blood group of both the recipient and the donor by testing the cells against known sera anti-A (group B) and anti-B (group A), and

*Refer to page 1008.

check these results by testing each of the blood sera, previously inactivated, against known cells (A_1 and B); (2) obtain the subgroup if the recipient is group A or AB; (3) cross-match the recipient's serum with the donor's cells, and the recipient's cells with the donor's serum; (4) determine the Rh factor. Blood cells are grouped by testing the corpuscles with known anti-A and anti-B sera; sera are grouped by testing against known A_1 and B cells. The exact technic is given on pages 1003 ff.

Caution: A serologic test for syphilis should be performed on all prospective blood donors to exclude syphilis. It is well also to bear in mind the possibility of transmitting malaria in blood transfusion. It is recommended, therefore, that a thick drop and blood film examination should be made of all donors to detect malaria as well as any blood dyscrasia which would make the donor objectionable. It is well not to transfuse blood of an allergic individual.

Attention has been called by several writers to the effect of sulfanilamide on the cross-matching of blood. The author has several cases on record at the Christian Hospital of St. Louis of difficulty in cross-matching in patients undergoing sulfanilamide treatment which were not related to disease but were apparently due to some peculiarity of the blood brought about by this therapeutic agent in the blood stream.

One example of the effect of sulfanilamide on blood grouping occurred with a group B donor whose blood was of a very high anti-A titer. This donor was given sulfanilamide therapy following appendectomy, and for a period of three months following administration of the drug, his blood serum agglutinated not only A and AB blood cells, but also group B cells.

Summary of Facts on A-B-O Blood Grouping.—All agglutination reactions depend upon two factors, the substance causing the reaction, and the substances acted upon. Isoagglutination, or agglutination of human blood cells by incompatible human blood serum, is this type of reaction. The substance causing the reaction is called *isoagglutinin* and is present in the blood plasma or serum. The substance acted upon is called *isoagglutigen*, and is present in human blood cells. The isoagglutinins in the serum are designated as anti-A and anti-B. Blood serum may have both of the isoagglutinins, or either, or it may contain neither. The isoagglutinogens in the red blood cells are designated by the large Latin letters A and B. Cells may contain both A and B, or either A or B, or may lack both, in which case they are called O. ("O" is now considered a blood factor. Group O blood cells are now considered to possess a corresponding agglutigen O.) The reaction between isoagglutinins and isoagglutinogens, resulting in the clumping of the cells, is called *isoagglutination*. The anti-A agglutinin in the serum causes the agglutination of blood cells containing the A isoagglutigen, while the anti-B agglutinin in the serum causes the agglutination of blood cells containing the B isoagglutigen. If either the isoagglutinin or the isoagglutigen is lacking, there can be no reaction; hence, in a transfusion, it is necessary to inject into the blood stream of the recipient blood cells containing no isoagglutigen which might be acted upon by the isoagglutinin in the serum of the patient.

Blood Factor C of the A-B-O System*¹

It is necessary to understand the difference between an agglutinin and a blood factor. An **agglutinin** is a substance on the surface of the red cell. There are many agglutinins on the human red cell shared by all members of the species, and others which are type-specific, such as the A-B-O agglutinins, the M-N-S agglutinins, the Rh-Irr agglutinins, etc. Agglutinins are antigenic. Injection of human cells into a sensitive subject results in formation of antibodies which are capable of either clumping or lysing the red cells. An agglutinin can cause formation of and combine with more than one kind of antibody. The properties of agglutinins which enable them to combine specifically with their corresponding antibodies are known as "**blood factors.**"[†]

Every agglutinin has multiple blood factors which characterize it. For example, agglutinin M has multiple factors **M_i**, **M_{ii}**, **M_{iii}**, etc.

The agglutinin A has two principal blood factors, **A** and **C**, while agglutinin B has two main blood factors, **B** and **C**. Corresponding to the three blood factors **A**, **B**, and **C**, there are three naturally occurring isoagglutinins, anti-**A**, anti-**B**, and anti-**C**. Thus the composition of the four blood groups can be summarized as in Table 96.

TABLE 96.—COMPOSITION OF THE FOUR BLOOD GROUPS

BLOOD GROUPS	RED BLOOD CELLS		SERUM ISOANTIBODIES
	AGGLUTININOGENS	BLOOD FACTORS	
O	*	*	Anti- A , anti- B , anti- C
A	A	A and C	Anti- B
B	B	B and C	Anti- A
AB	A and B	A , B , and C	None

"O" is now considered a blood factor.

If group O serum is titrated for anti-A and anti-B, and then absorbed with cells of group A, there is usually appreciable reduction in the titer for B cells; absorption with cells of group B will usually reduce the titer for A cells. If, however, one pools anti-A and anti-B sera and then absorbs such pooled sera with A cells, the anti-B titer is not reduced, nor is the anti-A titer reduced if such sera are absorbed with B cells. Thus it seems reasonable that group O serum contains a third isoagglutinin, anti-**C**, specific for a blood factor **C** shared by agglutinins A and B.

It is relatively easy to prepare high-titered anti-A immune rabbit sera free of the **C** antibody, but anti-B immune sera are generally of considerably lower titer and contain a good deal of anti-**C**, which is difficult to remove by absorption. Thus, if antisera from immunized rabbits are used for blood grouping, many group A individuals will be incorrectly classified as group AB due to traces of anti-**C** agglutinins in the anti-B reagent, which will clump both A and B cells.

There is evidence of the existence of blood group C, with an incidence of 1 in 60,000 individuals. In a case cited by Wiener,² the red cells of the indi-

*Agglutinin O and factor O are omitted for the sake of simplicity.

†To avoid ambiguity, Wiener has proposed the use of bold-face type for symbols representing blood factors and their specific antibodies, regular type for agglutinins and phenotypes, and italics for the genes and genotypes.

¹After Wiener, A. S.: *Ann. Eugenics* 18: 1-8, 1953.

²*Ann. Eugenics* 18: 1-8, 1953.

vidual reacted as group O in tests carried out in the usual way with anti-A and anti-B grouping sera, while the serum agglutinated all cells of groups A and B, but not of group O. In addition, the cells are strongly agglutinated by *all* group O sera. Group O sera contain the isoagglutinin anti-C not present in sera of other blood groups, which seems to prove that his blood belonged to rare group C. The serum of this individual contained both anti-A and anti-B isoagglutinins.

Nonspecific Agglutination

False Negative Reactions.—If testing sera are of high titer, the reactions are so definite with the open slide method that it is practically impossible to mistake them. Using the protein media or whole blood technic, these might be reported negative if the specimen is not spread thin enough on the glass slide and if the drop of saline is not added at the end of the time limit for the reaction. When testing unknown sera against known cells, the antibody concentration is not controlled, and weak positive reactions might occur. The same is true in cross-matching, where the drops must be searched diligently for slight clumping. Likewise, antibody titer gradually decreases in ampuled sera, so that controls must always be made before using such testing sera.

Another source of error may be low sensitivity of agglutinogens, which is seen when testing blood of newborn infants or at times when using preserved blood suspensions. One of the most common errors is to mistake subgroup A₂B for blood group B, due to the weak reactivity of the A agglutino-gen in such blood. The use of high-titered anti-A sera will obviate such results.

Pseudoagglutination.—Pseudoagglutination is the false appearance of agglutination which is not true agglutination at all, but usually rouleaux formation of the red blood cells. It simulates true agglutination. It is produced by high concentration of serum and high temperatures. It occurs on the slide test because of the low dilution of the serum and because the large surface favors evaporation and concentration of the serum. Serum from patients with rapid sedimentation rates will produce rouleaux formation with any red blood cells, including their own.

One must be cautious in using gum acacia, gum tragacanth, and gelatin to avoid pseudoagglutination. Care must be exerted in reading reactions where trypsinated cells are used. A negative control prepared just like the test must always accompany such tests.

The difference between pseudoagglutination and true agglutination can be readily seen under a microscope. In rouleaux formation, the red blood cells are arranged in piles like coins. By adding a few drops of saline, rouleaux formation or pseudoagglutination will usually disappear, while true agglutination either is not affected by this procedure or at times is made even more apparent.

Bacteriogenic Agglutination, the Huebner-Thomsen or Panagglutination Phenomenon.—Bacteriogenic agglutination is a phenomenon unrelated to isoagglutination, and results from bacterial action. It does not occur when perfectly fresh blood and serum are used. It is seen in some samples of stored

blood where the cells are clumped by any normal human serum, including that of the individual from whom the blood was derived, and also by group AB serum.

Bacterial contamination also affects blood sera, which then cause agglutination of red cells of several groups as well as their own groups. It may be avoided by using only freshly drawn blood.

Autoagglutination.—Autoagglutination is a condition in which the serum agglutinates its own red blood cells, due to the presence of an absorbable agglutinin in the serum and a corresponding agglutinogen in the cells. Autoagglutinins act not only on the subject's own cells, but also on the cells of all other human beings, regardless of blood group, and are therefore panagglutinins. The reaction occurs usually in tests made at low temperature. It usually disappears upon warming and reappears upon chilling.

In certain pathologic conditions, the autoagglutination titer is considerably increased and the reaction may occur at room temperature. Such conditions are atypical pneumonia, paroxysmal hemoglobinuria, syphilitic or hypertrophic cirrhosis of the liver, hemolytic icterus, Raynaud's syndrome, trypanosomiasis of man and animals, severe anemias, and other conditions. These autoagglutinins at room temperature are a source of error in blood grouping, especially when the titer is high. In some cases it is impossible to perform a red blood count, as pointed out by Gradwohl in Hodgkin's disease and febrile tuberculosis.

It is suggested that whenever a patient's group is determined as AB, the serum be tested against A and B cells. This is a good practice for all blood groups, and is recommended in this chapter as a routine test.

The serum can be freed of the interfering autoagglutinins by separating it from the cells at 0° to 5° C., at which temperature the blood cells absorb the autoagglutinins. The cells should be washed with warm saline (37° C.) before cross-matching.

"Cold" Agglutination.—Cold agglutination is due to the taking up of adsorbable antibodies in the cooled state. Since cold agglutination occurs in human bloods exposed to temperatures from 0° to 15° C., and is prevented by higher temperatures, this factor must be borne in mind when conducting tests.

Secondary Coagulation Simulating Agglutination.—Tests made with unwashed cell suspensions often show secondary coagulation, or the presence of a clot. This does not occur with washed cell suspensions or when inactivated sera are used for the tests.

False Agglutination by Umbilical Cord Sera.—Wiener observed that some cord sera produced a phenomenon resembling true agglutination. While testing a series of cord sera with A and B blood cell suspensions by the open slide method, he found that when the slide was tilted back and forth the cells came together in clumps, but that these clumps broke up again if the slide was allowed to remain at rest. The process could be repeated indefinitely. It was demonstrated that Wharton's jelly from the umbilical cord was responsible for the phenomenon.

Isohemolysis

Certain unheated blood sera, when mixed with incompatible blood cells, will dissolve them instead of causing agglutination. This reaction occurs between sera of human blood and human blood cells and is not to be confused with hemolysis of certain blood cells by specific amboceptor sera. This reaction is due to isohemolysins normally present in the sera and not caused by any pathology in the individual. The isohemolysins correspond in specificity to the isoagglutinins normally found in human sera. Thus it is not unusual for serum of group O to hemolyze the blood cells of group A, or even B or AB. The sera of group AB individuals do not contain isohemolysins. Usually agglutination of the cells occurs first, followed by hemolysis. This is not necessarily true, for we have frequently observed the dissolving of erythrocytes without preliminary agglutination. In one instance, a group B serum caused hemolysis of both A_1 and A_2 cells without first agglutinating them when the serum was unheated. After inactivation, this same serum gave a titer of 1:80 against the A_1 cells, but required 10 minutes to agglutinate the A_2 cells.

Isohemolysis can be prevented by inactivating the serum at 56°C . for 30 minutes to destroy the natural complement. The blood drop in which isohemolysis occurs can be observed in the process of clearing even by macroscopic examination. The orange-colored suspension will be seen to clear and will take on a reddish cast. Observation under the microscope often will disclose some erythrocytes not yet hemolyzed.

Isohemolysis is important because it may mask isoagglutination, and if unnoticed may lead to incompatible blood transfusions. This would occur in cross-matching when blood from a different group is given to the recipient. It is overcome in the usual grouping test by inactivating A and B antisera.

TECHNIC OF BLOOD GROUPING¹

1. Reagents.—

(Method of Unger)

The following testing sera are needed for complete blood grouping tests for transfusions, including sensitivity testing for Rh-Hr antibodies, the A-B-O groups and M-N types, the antiglobulin (Coombs) test, and conglutination tests. All sera used for blood grouping work must meet the requirements for standards of potency (high titer) of the National Institutes of Health (NIH).¹

Anti-A serum	Anti-Rh ₀ conglutinating (slide) serum
Anti-B serum	Anti-Rh ₀ ' conglutinating (slide) serum
Absorbed B (anti-A ₁) serum	Anti-Rh ₀ '' conglutinating (slide) serum
Anti-M serum	Anti-hr' conglutinating (slide) serum
Anti-N serum	Anti-hr'' conglutinating (slide) serum
Coombs serum (antihuman globulin)	Anti-Rh ₀ saline agglutinating (tube) serum
30% bovine albumin (Armour)	Anti-rh' saline agglutinating (tube) serum

¹All blood testing sera, as well as whole blood and other fractions, are considered biologics under the Biologics Law. They must be licensed, prior to commercial distribution, by the National Institutes of Health, United States Public Health Service.

Witebsky group A
and group B
specific substance

Anti-rh'' saline agglutinating (tube) serum

Anti-hr' saline agglutinating (tube) serum*

Anti-hr'' saline agglutinating (tube) serum*

The following cell suspensions must be prepared daily.† They are usable for 24 hours. Prepare both saline-suspended untreated and trypsinated cells of the various blood groups:

Group O Rh₂Rh₀

Group O Rh₁Rh₁

Group O Rh₂rh

Group O rh'rh

Group O rh''rh

Group O rh

Group A₁ (rh)

Group B (rh)

Additional reagents needed are:

Trypsin, Difco 1:250

Physiologic (0.85%) saline

Phosphate buffer, pH 7.41, M₆15, Eimer and Amend

2. Equipment.—

The list of equipment used here was compiled from that used in Dr. Lester J. Unger's laboratory in New York City (New York University-Bellevue Medical Center, Blood Bank).

Microscope containing a scanning lens, 32 mm. 0.10 strain-free. If this is not available, simply remove the front lens attachment from the low-power objective of a microscope, and use the remaining lens system.

Electrically controlled water bath, which should be kept covered at all times.

An angle centrifuge. That made by Ivan Sorvall,‡ 27-tube head, with 18 holders in the outer circle and 9 on the inner, is excellent. The Phillips-Drucker (St. Louis, Mo.) centrifuge is also recommended (Fig. 231).

Racks for small tubes. We recommend those made by Standard Scientific Corporation, Catalog No. 95222, with 4 rows of 10 holes per row. The top and middle sections have $\frac{5}{8}$ inch holes, while the bottom of the rack has small ($\frac{5}{16}$ inch) holes.

Viewing box (Clay-Adams, etc.) (Fig. 232).

A Brewer automatic pipetting machine is excellent but not absolutely essential.

Ordinary glass slides, 3 in. by 1 in.

Glass slides 3 in. by 2 in.

10-cell Boerner slides, 57₈ by 180 mm.§

Clay-Adams 12-ring slides, containing baked-on red enamel rings.

Wooden applicators.

Droppers and rubber bulbs.

Capillary droppers.

Soda straws, to use in place of droppers (in addition to droppers listed above).

Gauze pads, 3 in. by 3 in., 12 ply.

Red wax pencil.

Blue wax pencil.

Cover glasses, $\frac{7}{8}$ in. square.

Tubes: 4 in. by $\frac{1}{2}$ in.

3 in. by $\frac{1}{2}$ in.

7.5 cm. by 8 mm.

B-D Vacutainer tubes for collecting specimens of blood.

*Not available (1955). Use Anti-hr'' slide serum.

†In a small laboratory, if all the cells are not available, use group O Rh₂, O rh, A₁, and B cells.

‡210 Fifth Ave., New York 10, N. Y.

§Made by Mercer Glass Works, Inc., New York.

Containers, about 250 c.c. capacity, of different shapes, for holding saline and waste saline during the blood grouping tests.

3. Special Anticoagulant Used for Blood Grouping.—

Ammonium oxalate.....	2 gm.
Potassium oxalate.....	3 gm.
Distilled water.....	100 c.c.

Put 0.2 c.c. in the *bottom* of each tube. Sterilize by dry heat.

Add 4.8 c.c. of venous blood and mix by inverting about 20 times to keep the blood from clotting. An excess of oxalate might cause false negative results in Rh testing.



Fig. 231.—The Comet centrifuge. (Courtesy Phillips-Drucker, St. Louis, Mo.)



Fig. 232.—Diamond viewing box. (Courtesy Clay-Adams Company, New York.)

4. Preparation of Plasma.—

Take blood as indicated in (3) above, using the special anticoagulant.

Shake to prevent clotting.

Centrifuge.

Remove enough plasma for cross-matching tests, sensitivity testing, and any other work required. Leave enough to resuspend the cells for the protein-media testing. See below. Exact method of removing plasma is given on page 1009, Fig. 237.

5. Preparation of 2 or 3% Cell Suspension in Physiologic Saline (Unknown Cells).—

Place about 1 c.c. of saline in a Kahn tube (3 in. by $\frac{1}{2}$ in.).

Dip a straw into the cells left over from step 4, and add to the saline, mixing in and out of the straw until a 2 or 3% suspension is obtained. This is judged by the color (tomato juice).

If clotted blood has been submitted, loosen the clot with a wooden applicator, centrifuge, remove the serum, use some of the cells shaken from the clot to make the saline suspension. Wash once in saline and resuspend to a 2% concentration with saline.

6. Preparation of Control Cell Suspensions.—**(a) Untreated Saline-Suspended:**

Prepare cell suspensions as outlined in (5) above.

Centrifuge to pack cells.

Remove supernatant saline.

Shake tube to loosen cells.

Add saline to a 2 or 3% suspension (color of tomato juice). This is referred to as washing once with saline.

(b) Trypsinated Saline-Suspended:

Trypsin must be prepared daily. Use Difco trypsin, 1:250.

Use the Eimer and Amend (or other brand) phosphate buffer solution pH 7.41, M/15.

Put a small amount of trypsin (about one-quarter inch in a Wassermann tube) into a small tube, 5 by ½ inch.

Add enough phosphate buffer to make a supersaturated solution.

Centrifuge. Remove the supernatant fluid and place it in another tube.

Wash a saline suspension of desired cells 4 times in saline.

Pack after the last washing by centrifuging and removing the supernatant fluid.

To 9 drops of packed washed cells

add 1 drop of freshly prepared trypsin.

Shake and place in a water bath at 37° C. for 1 hour, shaking every 15 minutes while in the water bath.

Wash once with saline. (Add saline, centrifuge, remove supernatant trypsin, add saline, shake.)

Resuspend to 5% concentration in saline.

If kept refrigerated, trypsinated-saline suspended cells can be kept for 1 or 2 days. If some hemolysis occurs, wash once with saline and resuspend cells in fresh saline.

(c) Cells Suspended in Own Plasma (Unger).—

Add cells from the whole blood to some of the plasma removed from the whole blood, until a 2 to 5% suspension has been reached.

For routine work, group A₁, group B, group O Rh_zRh₀* cells are used; for titrations, group O Rh₁Rh₁, group O Rh₂rh, group O rh'rh, group O rh''rh, group O rh.

7. Preparation of Protein Media Cells Suspended in Their Own Plasma (C₅₀), 50% Suspension.—These will be referred to as "Whole Blood."

After centrifuging whole oxalated blood, remove part of the plasma until there are equal parts of plasma and cells.

Shake to resuspend the cells.

Whole blood is used for slide Rh testing, both with unknown and control blood, and for testing for A-B-O factors by the rapid slide method.

8. Using Droppers to Maintain a Standard Drop.—

In order to maintain standard drops throughout a test, and also to save time and equipment, the following method, as suggested by Unger, can be used.

Have three containers, approximately 250 c.c. capacity, each of a different shape so as not to confuse them. One is for saline for use in the test, one is for rinsing saline, and one is for discarded saline, cells, and plasma.

(a) Discharge material left in the dropper into the waste container, wiping off the outside of the dropper with a gauze pad.

(b) With the bulb still squeezed, pick up a dropperful of rinsing saline and discharge it into the waste container, and continue squeezing the bulb.

(c) Wipe off outside of dropper, and, still squeezing the dropper, pick up another dropperful of rinsing saline, discharge it into the waste container, and continue 5 or 6 times. Be sure to wipe off the outside of the dropper.

*Agglutinated by all 5 antisera: Anti-rh', Anti-rh'', Anti-Rh₀, Anti-hr', and Anti-hr''.

(d) With the bulb still squeezed so as not to admit anything from the dropper into the clean saline, pick up a dropperful of saline from the saline for use, and use what is needed, discharging the excess into the waste container. Always hold the dropper at the same angle when measuring drops for a test. Do not admit bubbles from a dropper, for this would be less than a full drop.

If handling serum or plasma by this method, discharge excess remaining serum or plasma from the dropper into the waste container and not back into the original specimen.

9. Preparation of Slides for Blood Grouping, Rh Testing, and Cross-Matching.—

Wax ring slides may be used (see below) for testing saline-suspended cells by the open slide method.

When using whole blood (protein media), prepare slides by drawing a large oval $1\frac{1}{2}$ in. by $\frac{3}{4}$ in. with a red wax pencil.

10. Preparation of Saline.—See page 2033.

CAUTION: When the sera are not in use, they must be kept refrigerated. Always protect the testing sera against bacterial contamination.

All work should be read by at least two technicians, without access to the records of each other. If there is any discrepancy in the results, the tests should be made again.

The cell suspensions used for the day's work should be prepared fresh each day at the same time, preferably by the same technician.

Preparation of Wax Rings

The use of paraffin ring slides has been alluded to. These are prepared in the same manner as for use in the Kline test. Prepare the slides by cleaning well to render them fat-free and make rings of wax with the usual contrivance furnished by the manufacturer, using melted paraffin to make the double circles of wax on the slide. The capacity of each one is about 0.05 c.c., so that in making the grouping test, small drops of serum and cell suspensions

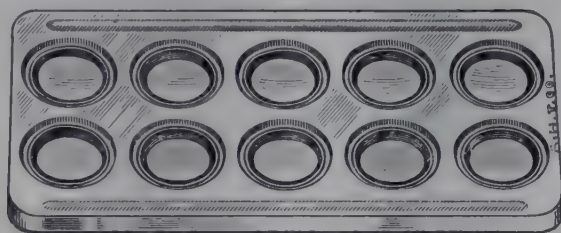


Fig. 233.—Ten-welled slide for blood grouping. (Courtesy Arthur H. Thomas Co., Philadelphia.)

should be used. Serum and cell suspensions are mixed by rotating the slide on its flat surface or tilting it back and forth 4 to 10 minutes, after which the reactions are read with the naked eye and with the microscope. By using a tray which can hold three of the paraffin ring slides, as many as 36 tests can be performed at one time.

An improvement over the wax rings on glass slides is that entailing the use of the Boerner micro test slides (Fig. 234). These test slides are 88 mm. long, 58 mm. wide, and 2 mm. thick, with rounded corners and twelve mold-pressed, raised rings, 15 mm. top diameter, in three rows of four each, numbered consecutively from 1 to 12. The bottoms of the cells are flat and slightly rounded at the periphery, which construction prevents capillary flow around the outer edge of the bottom and insures thorough shaking of the drop. It also permits rapid macroscopic and microscopic examination. While they were designed

for use in the Boerner-Jones-Lukens microflocculation test, they are very satisfactory for blood grouping procedures. A case of wood, with hinged lid, may be purchased from Arthur H. Thomas Company to house the slides, keeping them clean and preventing breakage, scratching, etc., when not in use. (See Fig. 235.) A more recently designed slide is seen in Fig. 233.

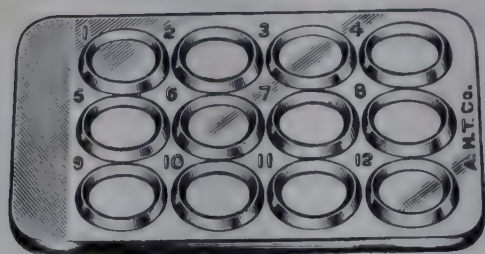


Fig. 234.



Fig. 235.

Fig. 234.—Boerner micro test slide. (Courtesy Arthur H. Thomas Company, Philadelphia.)

Fig. 235.—Case for holding Boerner micro test slides. (Courtesy Arthur H. Thomas Company, Philadelphia.)



Fig. 236.—Boerner electric rotating apparatus. (Courtesy Arthur H. Thomas Company, Philadelphia.)

Blood grouping tests are facilitated by using the Boerner electric rotating apparatus. (See Fig. 236.) This gives perfect shaking of blood grouping slides and has an automatic cut-off timing device, with speed control rheostat and motor with worm reduction gear and driving mechanism which rotates the platform in a horizontal plane so that it circles an area $\frac{3}{4}$ inch diameter to simulate hand rotation.

Outline of Blood Grouping Tests Made Routinely on Patient and Donor

(For other tests, refer to pages 1060 ff.)

1. Test for A-B factors on the patient's cells (and even if blood bank blood is available, on the donor's cells), using a 2 or 3 per cent suspension of cells in saline against known anti-**A** and anti-**B** sera.

2. At the same time, run controls on the antisera, using known group A₁ and group B, preferably type rh, cell suspensions in saline. The anti-A serum must agglutinate A cells but not B, and the anti-B serum must agglutinate B cells but not A.

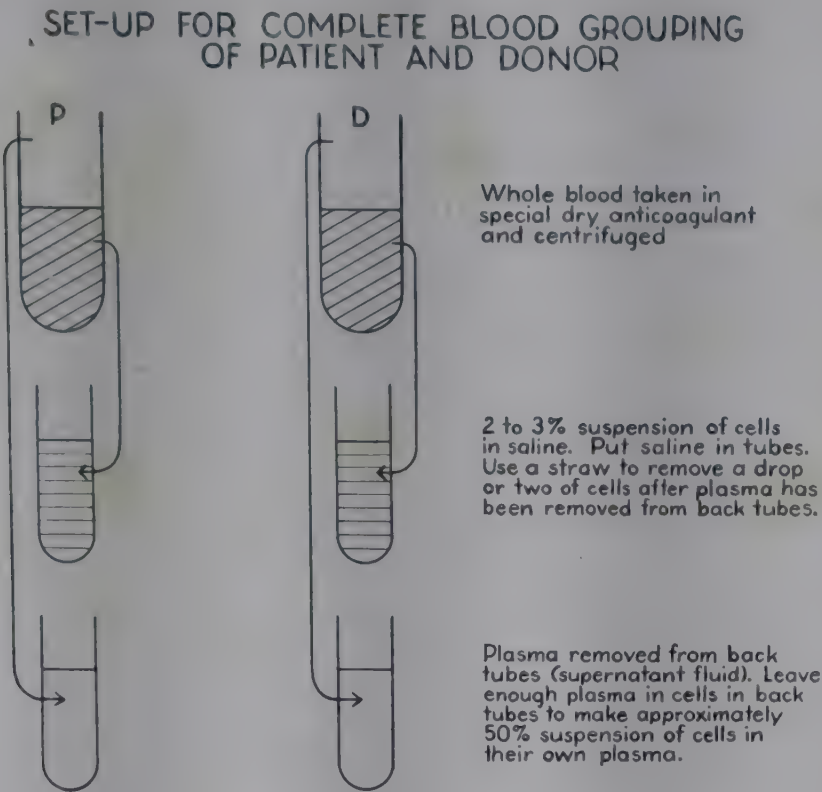
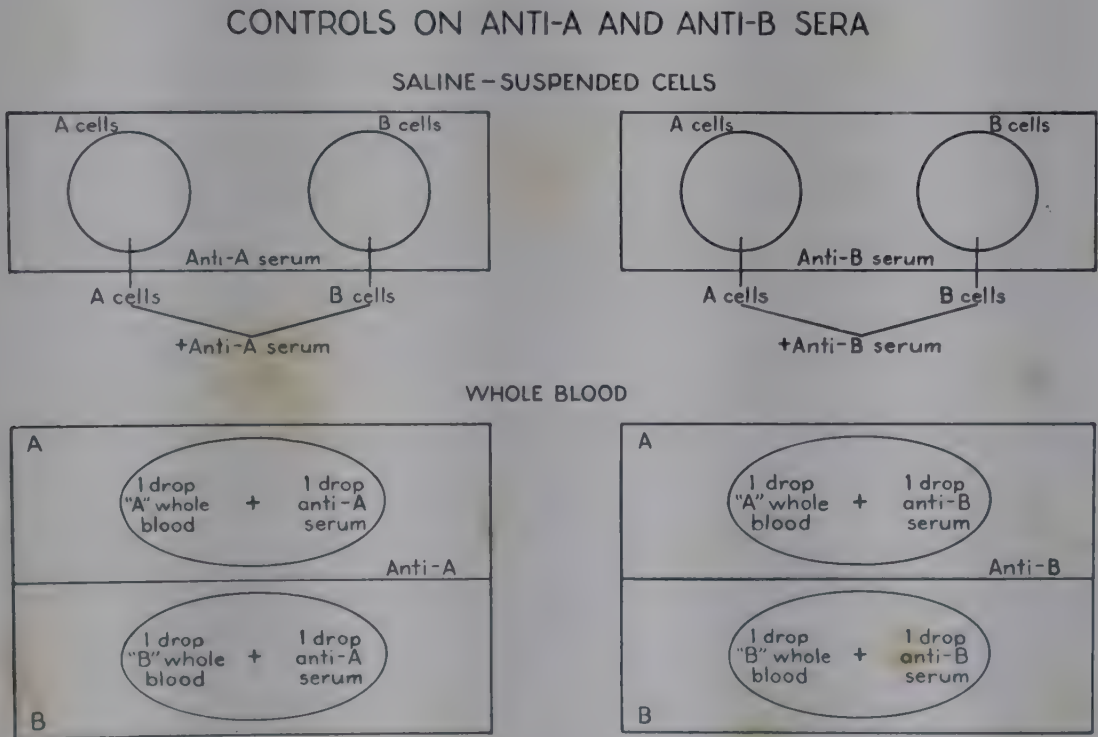


Fig. 237. (After Unger.)



3. Test the plasma (serum) of the patient (and the donor even if blood bank blood is available) against known group A₁ and group B cells suspended in saline. If isohemolysis occurs, inactivate the serum (plasma) and re-test.

4. Test the whole blood of the patient (and donor even if blood bank blood is available) against anti-A and anti-B sera, using the open slide method.
5. If the results in 1, 3, and 4 differ, repeat the tests; an error has been made.
6. Subgroup all A's and AB's by testing the cells against absorbed anti-A serum. This is not essential for ordinary blood transfusion work.
7. Test the patient's whole blood for the **Rh₀** factor by the slide agglutination test, using anti-**Rh₀** conglutinating (slide) serum. If the result is negative, consider the patient *Rh-negative*, and use only *Rh-negative* donor blood, even though the patient's blood might contain the **rh'** and **rh''** factors. Proceed to step 9 whenever possible.
8. Test the donor's whole blood against anti-**Rh₀**, anti-**Rh'**, and anti-**Rh''** conglutinating (slide) sera. If the blood is positive by any of these three, DO NOT GIVE IT TO AN Rh-NEGATIVE PATIENT. If using blood bank blood where all the tests are run, it is usually possible to obtain a type rh (triple Rh-negative) donor.
9. If the patient is Rh-negative by the slide test, perform an Rh tube test, using the washed cells, 2 or 3 per cent suspension in saline, against known anti-Rh saline-agglutinating (tube) sera. Test for **Rh₀**, **rh'**, **rh''**, and the **Rh₀** variant (**Rh₀**). If all are negative, the patient is type rh (triple Rh-negative), and thus **hr'** and **hr''** positive.
10. Cross-match (a) the donor's cells against the patient's serum (plasma)
(b) the patient's cells against the donor's serum (plasma)
using the cells suspended in saline, incubating in a water bath, reading macroscopically and microscopically. If the results of this test are negative, make an antiglobulin test on the cell sediment. See 12 below. If the results are positive, reject the donor.
11. Make an open slide protein media (conglutination) cross-matching test, using (a) donor's whole blood against patient's serum (plasma) and (b) patient's whole blood against the donor's serum (plasma).
12. At the same time as the cross-matching tests are run (10, above), make an Rh-sensitivity test, using the patient's serum (plasma) against group O Rh_zRh₀ cells (cells which are agglutinable by anti-**Rh₀**, anti-**rh'**, anti-**rh''**, anti-**hr'**, and anti-**hr''** sera):
 - (a) untreated and suspended in saline
 - (b) trypsinated and suspended in saline
 - (c) 2 or 3 per cent suspension in their own plasma.
13. It is well to make sensitivity tests of the donor's serum or plasma.
14. If any of the cross-matching tests are positive, reject the donor.
15. If the donor's plasma shows sensitivity to the Rh-Hr factors, reject the donor.
16. In all cases of pregnancy, make a sensitivity test on the mother's blood, and if positive, titrate for antibody concentration. Repeat the titration throughout the pregnancy as requested by the physician in charge of the case. (See page 1073.)
17. In unexplained erythroblastosis fetalis, test mother's serum for antibodies other than the Rh-Hr antibodies, and in the presence of an A-B-O incompatibility, titrate the A and B antibodies.

CONTROLS ON Rh ANTISERA

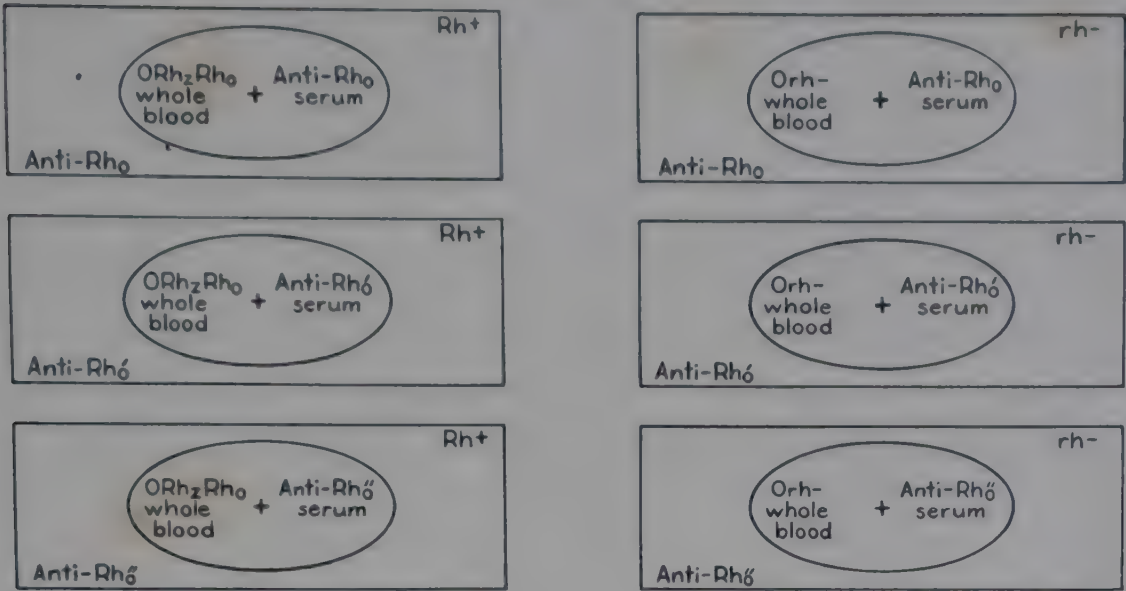


Fig. 239.

TESTS ON PATIENT AND DONOR

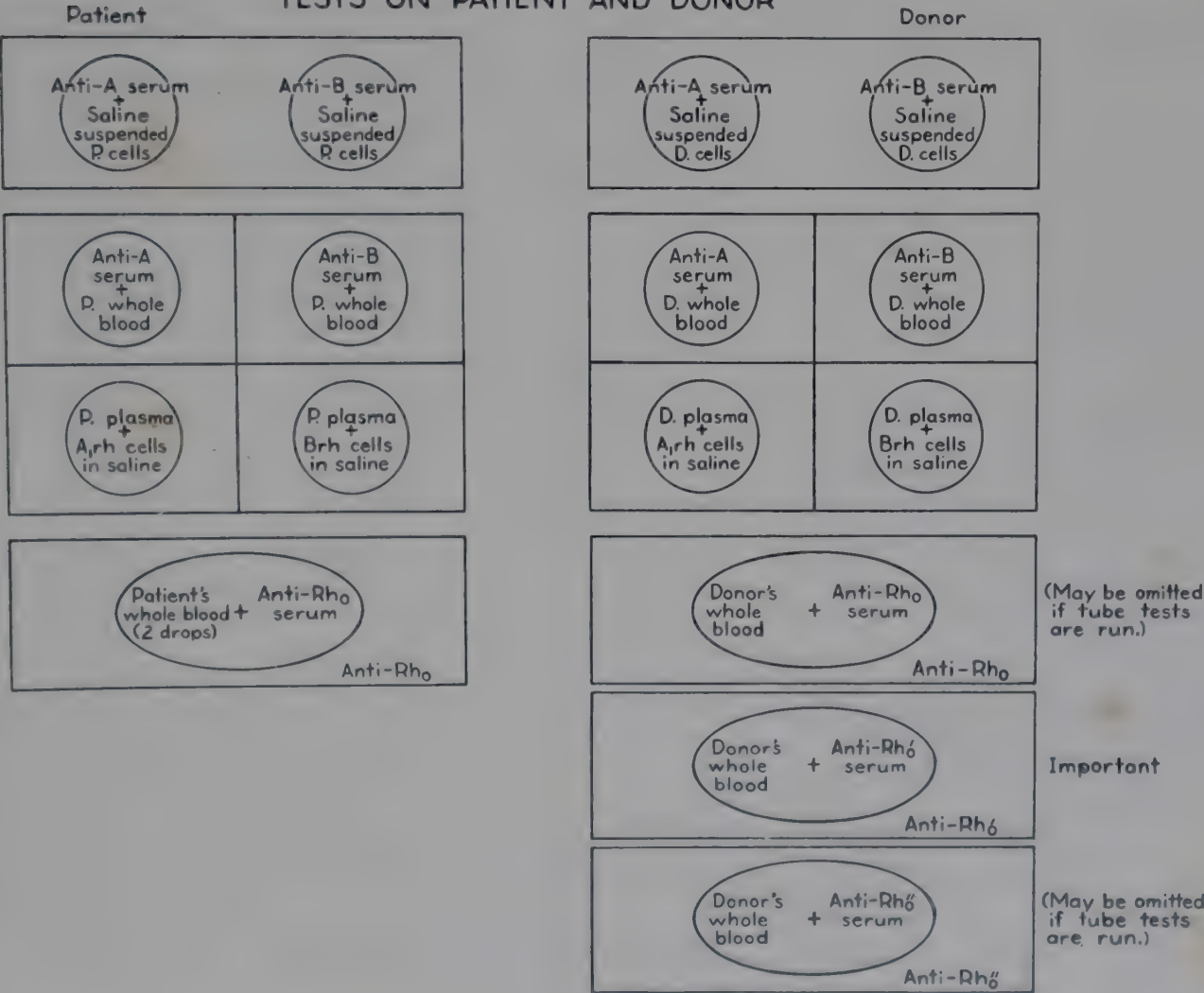


Fig. 240.

(I) Testing Unknown Cells to Determine Blood Group

(A) Using Saline-Suspended Cells.—

Controls

Have on hand glass slides containing two wax rings. (See Fig. 238.)

Label the left side “A” cells and the right side “B” cells.

Label the first slide “anti-A” serum and the second slide “anti-B” serum.

Place 1 drop of group A₁ cell suspension in the left-hand ring of each slide, and 1 drop of group B cell suspension in the right-hand ring of each side. (See page 1005 for preparation of cell suspensions.)

Add 1 drop of anti-A serum to each ring of the first slide, and 1 drop of anti-B serum to each ring of the second slide. Take care that the tips of the droppers do not touch the cells.

Mix and rotate, examining macroscopically and microscopically, under the low power without the Abbé condenser, at the end of five minutes, and again after eight minutes. Add a drop of saline to each ring before taking the final readings. Results expected are seen in Table 97.

TABLE 97

	“A” CELL SUSPENSION	“B” CELL SUSPENSION
Anti-A serum	+ (Agglutination within 45 sec. to 3 min.)*	- (No agglutination)
Anti-B serum	- (No agglutination)	+ (Agglutination within 45 sec. to 3 min.)*

*Good sera usually cause clumping within 10 seconds.

Agglutination must be + + +, and lack of agglutination must **not** be doubtful (see description below and page 1016). Anti-A serum must agglutinate the A cells but not the B, while the anti-B serum must agglutinate the B cells but not the A.

If the proper results are obtained, grouping of the cells of the donor and the recipient may be undertaken, using these anti-A and anti-B sera. If agglutination does not take place properly, or if the results are not correct in any way, discard the sera and obtain fresh reagents.

The Actual Test

Using a slide with two wax rings, label the left side “anti-A” and the right side “anti-B.” Place the individual’s name or laboratory number in the center of the slide for identification. (See Fig. 240.)

Place a drop of the unknown cells in each ring.

Add 1 drop of anti-A serum to the left side and 1 drop of anti-B serum to the right side. Take care that the tips of the droppers do not come into contact with the cell suspensions.

Mix and rotate, examining macroscopically and microscopically (under low power without the condenser). Record a positive reaction whenever it occurs, but wait eight minutes before recording a negative. At the end of the eight minutes, add a drop of saline to each ring, rotate thoroughly to mix, and read again. Record agglutination as a positive reaction and lack of agglutination as a negative reaction. If there is any doubt about the reaction, repeat the test.

Agglutination occurs as brick red clumping of the cells, the clumps being completely separate from each other. Lack of agglutination is simply the homogeneous suspension of the cells as they appear when suspended in saline. Do not confuse settling of the cells and rouleaux formation of the reds with true agglutination.

True agglutination is not affected by the addition of saline, while rouleaux are broken up with the addition of saline. See pages 1001 ff. for a discussion of false reactions. Avoid working in drafts or where cold air currents can blow over the open slides.

Determine the blood group by consulting Table 98.

TABLE 98. IDENTIFICATION OF BLOOD GROUPS, USING UNKNOWN CELLS

UNKNOWN CELLS PLUS ANTI- A SERUM	UNKNOWN CELLS PLUS ANTI- B SERUM	GROUP
-	-	O
+	-	A
-	+	B
+	+	AB

- = no agglutination; + = agglutination.

(B) Using Cells Suspended 50 Per Cent in Their Own Plasma (Whole Blood).—

Controls

Prepare slides as described under the actual test. (See Fig. 238.)

Label the slides A cells + anti-A, B cells + anti-A, A cells + anti-B, and B cells + anti-B.

Place about $\frac{1}{2}$ drop of known A whole blood in the slides labeled "A cells," and the same quantity of B whole blood in the slides labeled "B cells."

Add 2 drops of anti-A serum to each slide labeled "anti-A," and 2 drops of anti-B serum to each slide labeled "anti-B."

Mix and spread each with a separate wooden applicator.

Place on a lighted viewing box and handle as for the actual test (below).

The reactions should be the same as those in Table 97; a positive reaction should occur within 1 minute. Take the final reading in 5 minutes, after adding a drop of saline on each slide. Do *not* read microscopically.

The Actual Test

Prepare slides, 3 in. by 2 in., by making red wax oval rings $1\frac{1}{2}$ by $\frac{3}{4}$ in., two such rings on each slide.

Label one ring "anti-A" and the other "anti-B."

Label with the name or laboratory number of the individual being tested.

Place the whole blood to be tested in each ring by just touching the slide with the wet straw so that only a fraction of a drop is placed on the slide.

Add 2 drops of anti-A serum (of very high titer) to its proper ring, and 2 drops of high titer anti-B serum to its proper ring.

Mix each ring thoroughly and spread with a different wooden applicator.

Place on a lighted viewing box, and tip downward. Allow to remain on the box for 5 minutes, rocking back and forth occasionally. At the end of 5 minutes, read for clumping or lack of clumping.

With the box tipped downward, add a drop of saline to the top of each ring, and let it run down into the test. If rouleaux are present, the saline will break them up, but true agglutination will remain.

Determine the blood group, after waiting another 3 minutes, rocking the box back and forth, by consulting Table 98.

If the results using the whole blood and the cell suspensions are the same, proceed with the test, using the plasma or serum as the unknown; if they are different, an error has been made, and the tests must be repeated.

(II) Testing Unknown Serum or Plasma to Determine Blood Groups

In order to determine the blood group using serum or plasma as the unknown, it is necessary to have at hand cell suspensions of known group A₁ and B bloods, preferably rh, washed once and resuspended to 2 or 3 per cent concentration in saline. See page 1006.

If time permits, inactivate the serum at 56° C. for 30 minutes to destroy complement, and prevent subsequent hemolysis if the serum contains an iso-hemolysin. If an isohemolysin is present in a serum along with the corresponding isoagglutinin, the opposing cells begin to clump, then dissolve, and the reaction is very difficult or impossible to read. Isohemolysins will not act except in the presence of complement, which is destroyed at 56° C. When the complement is destroyed, the isoagglutinin acts in the usual manner, being thermostable, and the usual agglutination reaction takes place.

Use a slide with two wax rings. Label the slide with the name or laboratory number of the unknown. Label "A cells" at the left side of the slide and "B cells" at the right side.

Place 1 drop of known A₁ cells in the ring labeled "A cells" and 1 drop of known B cells in the ring labeled "B cells." (Fig. 240.)

Add a drop of the unknown serum or plasma to be tested to each ring.

Mix and rotate in the usual manner. Read a positive reaction as soon as it occurs, but do not take a final reading on a negative reaction until 8 minutes have passed. Read all reactions both macroscopically and microscopically. Agglutination may be strong or weak, depending upon the concentration of antibodies in the unknown serum.

Do not wait too long before reading, or surface drying takes place and pseudoagglutination may occur.

Add a drop of saline to each ring, mix again, and take a final reading under the microscope.

Determine the blood group of the unknown by consulting Table 99.

TABLE 99

A ₁ CELLS PLUS UNKNOWN SERUM	B CELLS PLUS UNKNOWN SERUM	GROUP
+	+	O
-	+	A
+	-	B
-	-	AB

Explanation: This is a test to determine anti-**A** and anti-**B** agglutinins in the unknown serum. Group O serum contains both anti-**A**, which agglutinates A cells, and anti-**B**, which agglutinates B cells. Group A serum contains anti-**B**, which agglutinates B cells but not A. Group B serum contains anti-**A**, which agglutinates A cells but not B. Group AB serum does not contain either anti-**A** or anti-**B** agglutinins, and therefore cannot agglutinate either A or B cells.

If the result in this test differs from the result obtained when testing the unknown cells against anti-**A** and anti-**B** sera, that is, if a different blood group is obtained, repeat both tests, but using different antisera and cell suspensions.

Preparation of Anti-A and Anti-B Testing Sera

Preparation of the Sera.—The testing sera must be of a high degree of potency (titer). It is a mistake to use testing sera that do not meet the standards of the N.I.H. It is a mistake to pool different lots of anti-**A** and anti-**B** sera and use them for testing purposes unless each of the pooled sera is of high titer. No anti-**A** serum should be used for testing purposes unless it will agglutinate A₁ cells at a 1:256 dilution, A₂ cells at a 1:128 dilution, A₁B cells at a 1:128 dilution, and A₂B cells at a 1:64 dilution. No anti-**B** serum should be used to test unknown bloods unless it will agglutinate B cells at a 1:256 dilution. These are the requirements of the National Institutes of Health. Unless one uses great quan-

tities of the testing sera, it is much better to purchase them already prepared and standardized. Be sure to examine for the expiration date, and use only those manufactured under special license, in order to avoid grave errors in blood grouping tests.

Select a group A_1 individual with a high titer serum, and without Rh antibodies. Use sterile precautions throughout the procedure of obtaining and preparing the serum. Remove the blood by venous puncture, either with a sterile 20 c.c. syringe or, if blood is desired in larger quantities, use the commercially available vacuum bottles without anticoagulant. If using the smaller quantity, eject the contents of the syringe, under sterile precautions, into a clean, dry, sterile 50 c.c. Rockefeller tube, and immediately cork. Label with the name of the person from whom derived, the group letter, and the titer. Allow the blood to clot with the tube in a slanting position. Loosen the clot by striking the tube against the palm of the hand, and centrifuge at high speed for 30 minutes.

Observing all sterile precautions, carefully remove the supernatant serum by means of a sterile dropper, placing it in a clean, dry, sterile tube labeled with the name of the person from whom derived, the group letter, and the titer. Immediately cork. Inactivate at 56° C. for 30 minutes to destroy the natural complement to prevent subsequent hemolysis, and to kill any contaminating bacteria.

Transfer this with sterile precautions to sterile ampules and hermetically seal. Label with the name of the donor, the group letter, the titer, and the date. Store in a refrigerator.

Save enough of the serum to re-test before using. Test against A_1 , A_2 , and B cells, suspended in saline, and also use the whole blood. The anti-**A** serum must agglutinate the A_1 and A_2 cells but not the B, while the anti-**B** serum must agglutinate the B cells, but not the A_1 or A_2 . If any other results are obtained, or if the reactions are not strong, or require too long a time to take place, do not use the serum.

Titration of the Sera.—It is necessary to titrate anti-**A** serum against A_1 , A_2 , A_1B , and A_2B cells, and anti-**B** serum against B cells, so that one does not use sera which are too weak to detect properly the blood groups of unknown blood. Weakly reacting sera will give false negative results in the grouping tests, which may lead to serious transfusion reactions. Three examples of each group of cells should be used.

In all titrations, serial dilutions of each serum are made, and each dilution tested against the opposing cells. The highest dilution of the serum which causes agglutination of its specific cells is called the titer. Many different methods of titrating sera have been proposed, but we prefer the method of Unger which follows.

Making the Dilutions for Titrations.—The sera should be inactivated at 56° C. for 30 minutes and allowed to cool to room temperature before dilution. Prepare serial dilutions of sera of 1:1 (undiluted), 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, and any other dilution desired or needed.

Use racks which hold 10 tubes per row. Rack up tubes 7.5 cm. by 8 mm., 10 per row, one row for each type of cells used. If titrating anti-**A** serum, four rows will be needed. If titrating anti-**B** serum, only one row will be needed.* These are the titration tubes. Refer to Fig. 254 for set-up for titrations.

At the same time, rack up two rows of Kahn tubes, 10 per row. These are the dilution tubes.

Use serum, not plasma, for titrations.

Place the serum in the first tube of the front row of Kahn tubes. Be sure that all tubes are properly labeled.

In all the back rows of dilution rack, add saline to fill the tubes. This will be used to rinse the dropper. There are now two rows of dilution tubes, the first row empty except for the first tube which contains serum, and a back row of 10 tubes filled with saline.

Put a drop of undiluted serum to be tested into the first tube of each row of the titration tubes.

Carry 10 drops from the first tube of serum to the second tube in the front row of dilution tubes.

Rinse the dropper with all the saline in tube 1 of the back row of dilution tubes, and remove the discarded saline tube from the rack. Wipe off the excess saline with a gauze pad.

*If anti-**B** is titrated against A_1B and A_2B , three rows will be needed.

Put 10 drops of clean saline, using the same dropper at the same angle, into each tube of the front row of dilution tubes, beginning with tube 2.

Use the same dropper throughout the procedure and hold it at the same angle.

Mix the contents of tube 2 thoroughly in and out of the dropper, taking care to avoid bubbles.

Put a drop of this 1:2 dilution of serum into the second tube of the titration racks, and carry 10 drops to tube 3 of the dilution tubes.

Rinse the dropper with all the saline behind tube 2, and wipe off the excess with a gauze pad.

Mix the contents of tube 3 (1:4 dilution) of the dilution tubes thoroughly, carry a drop to tube 3 of each titration row, and 10 drops to tube 4 of the Kahn tubes for dilutions.

Again rinse the dropper, using the saline in the tube behind tube 3, and continue the dilutions and disposal of drops as before, until all the dilutions have been made and the titration racks contain 1 drop of the respective dilution per tube: 1:1 in the first tube, 1:2 in the second, 1:4 in the third, 1:8 in the fourth, 1:16 in the fifth, etc. See Fig. 254.

Add 1 drop of the opposing cells* suspended in saline to each tube, using the same dropper after rinsing thoroughly with saline as directed above. This insures the same size drop for both serum and cells.

Leave for 2 hours at room temperature. Tip the rack so that the bottoms of the tubes are visible and take bottom readings. Negative reactions run down in a pear-shaped formation. Agglutination reactions usually remain round.

Flick the tubes and gently spread the contents over the tube, and read under a scanning lens of a microscope. Or remove a small amount of sediment from each tube and examine under a microscope.

Reading Results.—It will be noted that the clumps of blood cells gradually become smaller as the serum becomes weaker. With some sera, one dilution of serum may show strong agglutinating powers, while the next dilution shows a clear negative reaction. Report as *three plus strong* (+++s) any complete agglutination; that is, there are large brick-red clumps clearly visible to the naked eye, with no unagglutinated cells present. A *three plus weak* (+++w) reaction would be complete agglutination, but with smaller clumps. A negative (-) reaction is absence of any signs of agglutination microscopically or macroscopically. Record reactions as +++s, +++w, ++s, ++w, +s, +w, ±, and -. The last dilution in which there is definite agglutination is considered the titer.

Examples of Titrations

1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	TITER (UNITS)*
++w	-	-	-	-	-	-	-	-	-	1½
+++s	+++s	++w	+s	+w	-	-	-	-	-	16
+++s	+++s	+++s	+++s	+++s	+++w	+++w	++s	+w	±	256
+++s	+++w	+++w	++s	+	+w	-	-	-	-	32

*The titer (in units) is the reciprocal of the highest dilution of serum giving a one-plus reaction.

Obviously, the first serum would be of no value for laboratory testing purposes. This blood would, however, yield ideal plasma, because of the low isoagglutinin content.

Subgroups and Groups A and AB

Von Dungern and Hirszfeld¹ first called attention to the fact that when a group B serum was absorbed with certain group A bloods until it lost the power of agglutinating the absorbing blood, the serum still agglutinated most other bloods of group A and group AB. They suggested a subdivision of groups A and AB. In 1923 this was further emphasized by Coca and Klein.²

*See page 1015.

¹Zeitschr. f. Immunitäts. 8: 526, 1911.

²J. Immunol. 8: 477, 1923.

This resulted in the recognition of the fact that there are subgroups of A and AB; namely, A_1 and A_2 , and A_1B and A_2B . In other words, in the sera of group B and group O individuals, there are isoagglutinins acting on the isoagglutino-gen A. According to the common concept this isoagglutinin is a single entity designated as anti-A. The so-called α or anti-A isoagglutinin is composed of a number of qualitatively different fractions, of which there are two main varieties; (1) anti-A isoagglutinin proper, reacting with both isoagglutinogens A_1 and A_2 with approximately equal intensity, and (2) isoagglutinin anti- A_1 , which reacts with isoagglutino-gen A_1 but practically not at all with isoagglutino-gen A_2 . Different B and O sera contain these two isoagglutinin fractions in varying quantities and proportions.* Two different B sera which react with A_1 cells at approximately the same titer may have different titers when tested against A_2 cells. If, for instance, such sera contain very much anti- A_1 , and very little of the common anti-A isoagglutinin, there might be a titer of 40 for A_1 cells and a titer of only 5 for A_2 cells. On the other hand, another serum which contains a large proportion of common anti-A isoagglutinin might have a titer of 20 to 40 for A_2 cells even though the titer for A_1 cells is no greater than that of the first serum. In some cases B serum may react distinctly on A_1 and practically not at all on A_2 cells. In the case of AB blood, the danger of using such sera for testing purposes is great, since the sensitivity of A_2 isoagglutino-gen in this group is greatly diminished and such blood is readily mistaken for group B. It is for this reason that every group B serum used for grouping purposes should be titrated against A_2 as well as A_1 cells. Since A_2 makes up approximately one-fourth to one-fifth of all group A individuals, it is manifestly clear that with weak anti-A testing sera 20 to 25 per cent of group A individuals may be erroneously diagnosed as group O.

It is, therefore, important that in all grouping tests one must subgroup all A's to determine whether they belong to A_1 or A_2 classification. After the regular grouping tests have been done, take out all the A's and determine which are A_1 's and which are A_2 's. This is carried out with what is known as absorbed B (anti- A_1) serum.

Preparation of Absorbed B (Anti- A_1) Serum.—Observe all sterile precautions throughout the entire process. If the serum is contaminated, it will lose its agglutinating powers and will give false results. Place a few c.c. of sterile 3.8% sodium citrate in a sterile 50 c.c. Rockefeller tube and replace the cork immediately. Withdraw blood with a syringe from the vein of an individual of subgroup A_2 and place with sterile precautions in the sodium citrate solution. Cork and mix well to prevent coagulation. Wash three times with sterile 0.85% saline (see page 2043 for the washing of blood cells), and pack by centrifuging. Discard the supernatant fluid after the last washing. These cells are the packed, washed A_2 cells. When possible absorb with pooled A_2 cells derived from a number of individuals.

Place group B (anti-A) serum, obtained under sterile precautions, in a 50 c.c. sterile Rockefeller tube, and inactivate at 56° C. for 30 minutes. Add one-third to one-fourth as much of the packed, washed, pooled A_2 cells. Cork and mix back and forth. Allow the mixture to stand for one-half hour at room temperature, inverting occasionally, and centrifuge to separate the serum. Remove and keep the supernatant fluid, which is absorbed B (anti- A_1) serum. It will usually be found that the serum recovered no longer reacts with A_2 cells, but still agglutinates A_1 cells distinctly.

*Wiener, A. S.: *Am. J. Clin. Path.* 9: 145, 1939.

To Test Absorbed B (Anti-A₁) Serum.—Have on hand three known A₁ and three known A₂ cell suspensions. These are prepared in the usual manner (see page 1005).

Place a drop of each cell suspension in a wax ring on a slide properly labeled and add a drop of absorbed B (anti-A₁) serum to each one.

Rotate and observe every 30 seconds under the low-power objective of the microscope until 5 minutes have elapsed. A₁ cells should be agglutinated within 1 or 2 minutes, while the A₂ cells should not be agglutinated at the end of the 5- to 10-minute period. If neither A₁ nor A₂ cells have been agglutinated, discard the serum. If the A₂ cells agglutinate in less than 5 minutes, repeat the absorption, using less A₂ cells than during the preceding absorption period, and reducing the time of absorption to guard against over-absorption. Test the serum again against known A₁ and A₂ cells.

Technic of Subgrouping A and AB Blood Cells (Using Absorbed B [Anti-A₁] Serum)

(1) **Controls.**—The reagent used for subgrouping groups A and AB cells is absorbed B (anti-A₁) serum. When using an absorbed serum it is necessary to limit the time between making the test and reading the reaction, since many absorbed sera are slightly underabsorbed and may give false reactions after a prolonged time period. In order to determine the *time element* in reading subgrouping tests, it is well to make controls on the serum just before using, testing it against known A₁ and A₂ cell suspensions. Prepare suspensions of known A₁ and known A₂ cells. Place a drop of known A₁ cells in a wax ring on a slide properly labeled, and a drop of known A₂ cells in another ring also properly labeled. Add to each drop one drop of absorbed B (anti-A₁) serum. Rotate and observe under the low power of the microscope until agglutination takes place, recording the time required to agglutinate A₁ cells. A₂ cells should remain unagglutinated for at least 8 minutes, but if the serum is not completely absorbed, they may eventually be agglutinated by the serum. Record the time the first small clumps appear in the A₂ cell suspension ring. The test on the unknown cells must be read within this time limit. For example, A₁ cells were agglutinated in 1½ minutes, but A₂ cells did not begin to agglutinate before 10 minutes. Read the test on unknown A or AB blood cells at the end of 6 or 7 minutes.

(2) **The Actual Test.**—On a glass slide containing one wax ring, labeled with the name of the unknown and the group letter (either A or AB), place one drop of the unknown cell suspension. Add 1 drop of absorbed anti-A serum and rotate. Read microscopically under the low power without the Abbé condenser. Read periodically but record the reaction at the end of the time limit determined in the controls. Report agglutination as positive and lack of agglutination as negative. A positive reaction means that the subgroup is A₁ or A₁B as the case may be. A negative reaction means that the subgroup is A₂ or A₂B. Tables 100 and 101 give these results in chart form:

TABLE 100		TABLE 101	
A CELLS PLUS AB-SORBED ANTI-A SERUM	SUBGROUP	AB CELLS PLUS AB-SORBED ANTI-A SERUM	SUBGROUP
+	A ₁	+	A ₁ B
-	A ₂	-	A ₂ B

Agglutinogens A₃ and A₄.—In 1935 Fischer and Hahn¹ reported the occurrence of individuals of group A whose blood gave only feeble reactions even with the most potent anti-A sera. Friedenreich, reporting on the same phenomenon, conceded that the weak reactivity of such bloods was due to a third sort of A isoagglutinogen; namely, A₃. This isoagglutinogen A₃ gives rise to two additional subgroups in group A and AB; namely, subgroups A₃ and A₃B, respectively. These subgroups can be detected without any special method by their weak reactions with anti-A sera. A₃ blood, like A₂ blood, is agglutinated by sera containing anti-A₂ isoagglutinin. A₃ sera contain anti-B

¹Ztschr. f. Immunitäts. 84: 177, 1935.

isoagglutinins just like ordinary group A sera, but, as in A_2 sera, anti- A_1 cold isoagglutinins may be present in these and in A_3B sera. The existence of the A_3 receptor may account for certain errors in blood grouping.*

Wiener and Silverman report an interesting case belonging to group A_3 . This individual belonged to group A but showed weak agglutination reactions with anti-A sera, both those obtained from group B human beings and those prepared by immunizing rabbits with human A blood. Blood of this case gave a very weak agglutination with anti-A sera while the A_2 cells as well as the A_1 cells gave prompt and marked agglutination.

Microscopically, this patient's blood showed many free cells. With weak anti-A sera, these writers stated that the patient's blood might fail to react. That she belonged to group A was further established by testing her serum against standard O, A_1 , A_2 , and B cells. With the B blood, prompt agglutination was obtained, demonstrating the presence of the anti-B isoagglutinin. No reaction occurred with O or A_2 bloods, but a weak reaction occurred with the test with A_1 cells. Therefore, this patient's serum contained, in addition to the anti-B isoagglutinin, an irregular isoagglutinin anti- A_1 . These observers felt justified in placing this blood in the subgroup A_3 .

An A_4 blood group has been described by Gammelgaard and Marcussen.¹ There is a weak reaction of the A_4 with B sera. A further serologic characteristic is a reaction with certain O sera and immune sera of rabbits, which is stronger than in B sera. An irregular agglutinin for A blood corpuscles was proved in the sera of 13 out of 15 cases. A_4 was found only once in routine examination of 60,000 blood samples. The presence of A_4 was proved in 24 persons of a family of 64 members. The importance of the A_4 group lies in a possible mistaken identification by reason of the very weak reaction, which is further lowered in combination with agglutigen B. The A_4 group will rarely be revealed in routine serologic examinations of sera; therefore, in transfusions it may easily happen that blood of the A_4 group instead of O group is used. The A_4 group is comparable in its importance in legal medicine to that of the A_3 group, provided the hereditary process is clarified.

Cross-Matching

After the blood group and Rh type of the patient have been established, and a donor of the same blood group and Rh type has been tentatively selected, cross-matching tests must be made. That is to say, one must match the donor's cells with the patient's serum and the patient's cells with the donor's serum, and there must be no signs of agglutination of the red cells. It is well to make sensitivity tests for Rh-Hr antibodies at the same time as the cross-matching tests are run. See page 1062 for the method.

Caution must be exercised in accepting blood grouping designations by other laboratories. Remember that the person making the transfusion is responsible for whatever accident may happen; therefore, all donors and recipients must be re-grouped or re-typed when they appear for examination or transfusion. This is in no way intended to criticize the work of other laboratories, but is simply a measure of precaution which each one must follow.

*Also see Wiener, A. S., and Silverman, L. J.: *Am. J. Clin. Path.* **11**: 45, 1941.

¹Gammelgaard, A., and Marcussen, P. V.: *Ztschr. f. Immunitäts.* **98**: 411, 1940.

Another procedure, which is mentioned only to be condemned, is to do cross-matching *alone*, omitting blood grouping.

Correct cross-matching results can be obtained only on bloods of the same group or subgroup. That is to say, one may match the cells of the so-called "universal donor," group O, with the serum of almost every other individual, but the reverse gives incompatible results. Since cross-matching does not usually detect Rh incompatibilities, it is necessary to make Rh tests on both patient and prospective donors and to carry out the conglutinin method.

From time to time it was claimed by some that cross-matching tests preliminary to transfusions were superfluous. It was erroneously believed that if the A-B-O blood grouping tests were properly carried out on the blood of the patient and donor, and if the results showed them of the same blood group, there would be no danger of a transfusion reaction. Unger found this to be incorrect.¹ Unger was the first to report that occasionally serum of a patient would clump cells of a donor even though they both belonged to the same A-B-O blood group, and that transfusion of such blood was followed by chills and fever which he attributed to the observed incompatibility. He called the antibodies responsible for the intragroup incompatibility "minor" agglutinins to distinguish them from the anti-A and anti-B agglutinins. He used a 2 per cent suspension of the cells in the individual's own plasma to carry out these cross-matching tests, although at that time it was customary to use saline-suspended cells. He had unwittingly carried out the "conglutination" test and detected the univalent antibodies (blocking antibodies) although he did not identify them. Most likely some of these may have been Rh antibodies.

With the development of blood banks, there has been a great increase in the number of patients transfused and the number of transfusions given to the same patient. These repeated administrations of blood expose patients to a great variety of antigens, and they may become sensitized, since it is practically impossible to transfuse a patient with blood that is identical with his own with respect to all blood factors. There are now known to exist more than 50,000 varieties of blood. Perhaps some day a complete blood grouping may become as distinctive as a thumb print, as predicted by Karl Landsteiner.

The following specimens are needed for proper cross-matching tests: 2 or 3% suspension of patient's cells in saline, 50% suspension of patient's cells in its own plasma (whole blood), and patient's serum or plasma. The same specimens are needed from each prospective donor.

(A) Tube Method of Cross-Matching (Fig. 241).—

Saline Agglutination Test.—

Use tubes 4 in. by $\frac{1}{2}$ in. for cross-matching.

Place in a rack, in two rows. Two tubes are required for cross-matching one donor's blood with one recipient's blood. If more than one donor is available, run two tubes for each donor.

Label the back tubes "DS," for donor's serum, and the front tubes "PS" for patient's serum. Identify each tube with the correct name or laboratory number.

Place 2 drops of the donor's serum in the back tube.

Place 2 drops of the patient's serum in the front tube.

Add 2 drops of patient's cells, suspended in saline, to the back tube.

Add 2 drops of the donor's cells, suspended in saline, to the front tube.

¹Unger, L. J.: J.A.M.A. 76: 9, 1921.

Mix and incubate in a water bath at 37° C. for ½ hour.

Centrifuge at 500 r.p.m. for 2 minutes.

Pick up a small amount of the sediment from each tube, with a straw or a dropper, and place on a properly labeled slide. Put a cover glass over each drop.

Examine under the low power of a microscope, looking for clumping or lack of clumping. If clumping of the cells has occurred, reject the donor. Examine the slides very carefully.

CROSS MATCHING AND Rh-Hr SENSITIVITY TESTING FOR ANTIBODIES IN SERUM

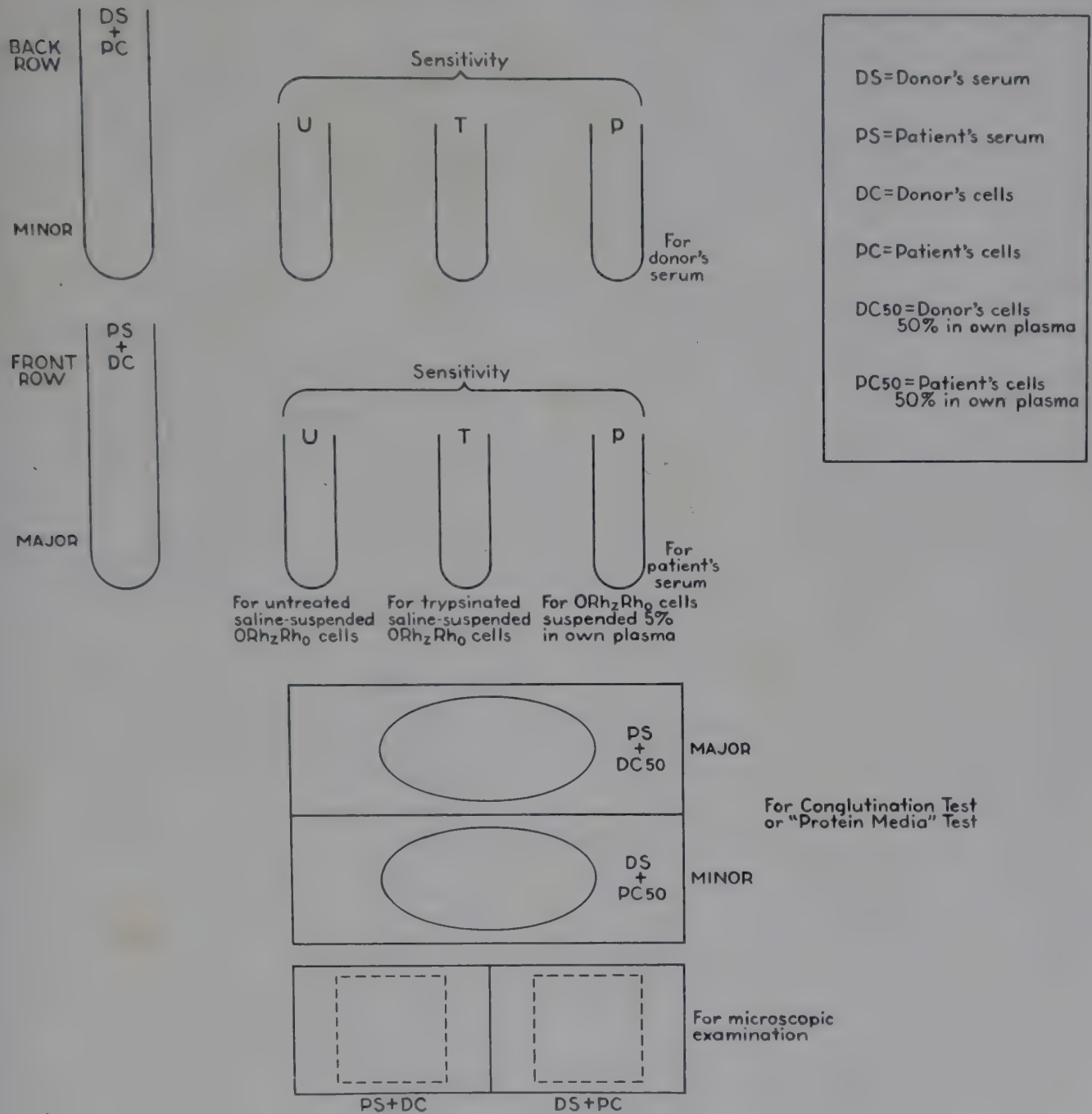


Fig. 241. (Unger.)

If the results are negative, examine the contents of the tubes under a scanning lens of a microscope, by spreading the sediment the length of the tube, with gentle rocking of the tube. If clumping appears, reject the donor.

This is a control over the slide reading.

If the results are all negative, proceed with a Coombs test.

Coombs Test (Antiglobulin Test).—

Coombs tests are made when the results in the cross-matching test are *negative*.

Shake the tubes, add saline to the capacity of the tubes, centrifuge, and remove the supernatant saline. This is the first washing.

Shake the tubes, again add saline, and recentrifuge. Remove the supernatant saline and discard. This is the second washing.

Repeat for a third washing.

Remove the saline, turn the tubes upside down, and pat against a gauze pad to remove all traces of the saline from even the lip of the test tube.

Gently shake the tubes to loosen the cells.

Add 1 drop of antihuman globulin or Coombs serum to each tube.

Mix. Incubate in a water bath at 37° C. for 5 minutes.

Centrifuge at low speed, 500 to 1,000 r.p.m., for 2 minutes.

Gently dislodge the sediment and read over a lighted viewing box and under a scanning lens of a microscope.

If any signs of clumping occur, reject the donor.

When A-B-O compatibility has been proved, this test detects isosensitization of the serum to one or more factors of the blood it agglutinates.

(B) Slide Method, Using Whole Blood (Protein Media, Unger) (Fig. 241).—

Prepare a slide 3 in. by 2 in., by drawing two large oval rings the length of the slide and half the width.

Label the upper ring "PS" and the lower ring "DS" (patient's serum; donor's serum). Identify with the name or laboratory number.

Place 1 drop of patient's serum and 1 drop of donor's whole blood in the upper ring.

Place 1 drop of the donor's serum and 1 drop of the patient's whole blood in the lower ring.

Mix each with a different wooden applicator, spreading over the entire area of the ring.

Place over a lighted viewing box and tilt back and forth for 5 minutes, observing for clumping. *Do not read microscopically.*

At the end of 5 minutes, tilt the box, add a drop of saline to the top of each drop, and let it run down the drop. This is to break up rouleaux which might have formed.

If there are any signs of clumping, reject the donor. If there is no clumping, the cross-matching tests are compatible.

Some laboratories prefer the conglutinin method of cross-matching to the Coombs test. However, this is not good practice because the conglutination method cannot detect anti-*Duffy*. This can be detected only by the anti-globulin method.

(C) Conglutinin Method of Cross-Matching, Wiener.*—

Carry out the test in 2 small Kahn tubes.

Place two drops of recipient's serum in a tube marked "RS."

Place two drops of donor's serum in a tube marked "DS."

Add two drops of 30% bovine albumin to each tube.

Add *one* drop of 2% suspension of donor cells in saline to the tube marked "RS."

Add *one* drop of 2% suspension of recipient cells in saline to the tube marked "DS."

Do not add more than one drop of cell suspension to either tube.

Incubate for 3 to 5 minutes at 37° C.

Centrifuge for 2 minutes at 1,000 r.p.m.

Dislodge the cell sediment very gently. Excessive shaking may break up agglutination. Observe for agglutination or lack of agglutination.

When A-B-O group compatibility is proved, agglutination in the tube marked "RS" indicates that the recipient is immunized against a blood factor of the donor cells. Further cross-matching with other donor bloods must be carried out until blood is found which will not be agglutinated by the recipient's serum. Before testing with other donor bloods, the Rh type of the recipient must be carefully determined.

When Rh-positive recipients are immunized to Rh-positive blood belonging to a different Rh type, and when Rh-negative recipients are erroneously designated Rh-positive and isoimmunized, incompatibility with the donor blood may be detected by this method.

*Wiener, A. S. *Am. J. Clin. Path.* 12: 302, 1942.

Simple Blood Matching

Blood matching is not recommended since it assumes that the donor belongs to a group different from that of the recipient. According to older ideas, group O was a universal donor and could be used to give blood to all four groups, while AB was the universal recipient and could receive blood in a transfusion from all other groups. It was considered safe for all groups to give blood to their own groups and to group AB, and for all groups to receive blood from their own groups and from group O. In the light of later investigations, this arrangement has been shown to be dangerous.

Simple blood matching is still used in some laboratories, but it is not recommended. This test is made by mixing recipient's blood serum with the donor's cells, and observing for signs of agglutination. If any clumps of blood cells are seen, no matter how small the clumps, the donor is rejected.

The P Factor

Landsteiner and Levine,* in 1928, described an agglutinin P in human blood, more frequently found in Negroes than in whites. They demonstrated this factor with immune rabbit serum. Occasionally a natural irregular anti-P agglutinin is found in human serum, which acts on the P agglutinin in human cells. The reaction is extremely weak but may sometimes be demonstrated if the test is made in small tubes and not centrifuged, but kept in a water bath at 20° C., shaken occasionally, then examined under the microscope.

Anti-P agglutinins have been found in the sera of certain animals, such as, rabbits, hogs, cats, and cattle.

Landsteiner and Levine concluded that the property P is not a single, well-defined entity, but a group of related agglutinogens.

Occasionally anti-P agglutinins are found in normal human sera and are of sufficiently high titer to be usable as diagnostic reagents. Anti-P sera have been prepared from selected sera from P-negative professional donors containing an exceptionally high titer of natural P agglutinins.

Tests for P agglutinin are at times helpful in special problems of incompatibility in blood transfusions. The tests have also been used in anthropologic investigations, and in serologic studies on individual differences in human blood.

The P agglutinin is inherited as a simple Mendelian dominant by a pair of allelic genes designated *P* and *p*, respectively. The tests can therefore be used in medicolegal cases of disputed parentage, though this is not recommended.

The names "type P" and "type pp" are used instead of terms P positive and P negative. There are also bloods containing a variant of the P agglutinin.

Genotypes of P are *PP* and *Pp*; of pp are *pp*. Approximately 75 per cent of Caucasoids and 98 per cent of Negroids have been found to belong to type P, while 25 per cent of Caucasoids and only 2 per cent of Negroids have been found to belong to type pp. The percentage of type P among Mexican Indians has been found to be 78.9.

*Landsteiner, K., and Levine, P.: J. Exper. Med. 47: 757, 1928.

AGGLUTINOGEN Rh IN HUMAN BLOOD

Rh-Hr Nomenclature*

Two principal methods of designation of the Rh-Hr types are currently in use, the original Rh-Hr nomenclature and the C-D-E symbols. Significantly, the nomenclatures interpret the same set of observations differently, and so both cannot be correct. The Committee on Medicolegal Problems* has analyzed the available information in order to resolve this problem. Their conclusion is that the C-D-E notations "make no allowance for the difference between a blood factor and an agglutinin, and make the tacit incorrect assumption that every agglutinin has but a single corresponding antibody. . . . The Committee therefore recommends that the C-D-E notations for the Rh-Hr types be discarded, and that the original Rh-Hr nomenclature be retained as the sole nomenclature for this blood group system."

Reports of post-transfusion reactions when bloods of homologous groups have been transfused, where preliminary tests have been made by experienced observers using reliable and potent sera, indicate that these reactions represent intragroup incompatibilities. Cases of this type have been observed with unusual frequency following primary transfusions given to pregnant or postpartum women. It is believed that these represent an example of isoimmunization.

Isoimmunization

Isoimmunization was first noted by Ehrlich and Morgenroth¹ in 1900. *Heteroimmunization* means immunization of a member of one species by a member of another species. *Isoimmunization* means immunization of a member of a species by another member of the same species. Isohemolysins were produced in the goat by injecting erythrocytes of other goats. In the Ehrlich and Morgenroth goat blood injections, the immune, not normal, isoantibodies hemolyzed the cells from the goats that furnished the original inocula and also the cells of some, but not all, other goats. The serum did not contain an autolysin; that is, it did not affect or combine with the cells of the animal in which the antibody was formed. Their observations showed the possibility that a considerable number of different antigens might exist in similar cells of different individuals in a single species and also showed that an animal will not ordinarily produce antibodies to antigens that are present in his own blood. An animal can form antibodies against some of his own proteins or other components of his tissues, for example, the substance of the crystalline lens, casein,² and an alcohol extract of brain,³ but these materials are blood-foreign and thus satisfy one of the primary prerequisites for antigenicity. Immune isoantibodies have been produced in other animals and fowls (Wiener⁴).

It is difficult to perform isoimmunization experimentally in man. Thomsen⁵ attempted to immunize men by intramuscular injections of incompatible blood, but no significant rise in isoagglutinative titer was observed. This apparent evidence against the isoantigenicity of the A and B group-substances is, how-

*From Medicolegal Applications of Blood Grouping Tests, Supplementary Report; A Report of the Committee on Medicolegal Problems, American Medical Association, prepared by A. S. Wiener, R. D. Owen, C. Stormont, and I. B. Wexler. In press, 1956.

¹Ehrlich, P., and Morgenroth, J.: Berl. klin. Wchnschr. 37: 453, 1900.

²Lewis, J. H.: J. Infect. Dis. 55: 168, 1934.

³Idem: J. Immunol. 41: 397, 1941.

⁴Wiener, A. S.: Blood Groups and Blood Transfusion, ed. 3, Springfield, Ill., 1943, Charles C Thomas.

⁵Thomsen, O.: Ztschr. f. Rassenphysiol. 2: 105, 1930.

ever, outweighed by the positive evidences which will be presented in reference to the Rh factor. Thomsen's negative result is probably attributable to the relatively small quantity of blood injected, or, possibly, the route of injection was influential in his negative results. Thalhimer⁶ reported a case which is an example of the isoantigenicity of group-substance B. Here, a boy received 300 c.c. of his father's blood; cross-matching showed no incompatibility but no tests were made to determine the two blood groups. Only a mild febrile reaction ensued. Eighteen days later, without further tests, the father again served as donor; an alarming reaction stopped the transfusion after 150 c.c. of blood had been introduced. Subsequently, the bloods were grouped; the son was found to be group O and the father group B. Although no agglutinative titers were determined, it is probable that the anti-B normally present in group O plasma (with anti-A) was too weak to be detected by the ordinary test, and that the primary transfusion of group B blood served as an immunizing stimulus leading probably to a marked rise in the titer of anti-B, which thus intensified the incompatibility. There is further evidence that A and B are isoantigenic in Jonsson's⁷ finding that the maternal isohemagglutinins anti-A and anti-B are of higher titer when the corresponding antigen, A or B (not present in the mother's blood), is present in the blood of the fetus than when it is absent (transplacental isoimmunization).

Wiener⁸ recorded two cases which illustrate isoimmunization of A₂ individuals to A₁ blood. In one, the antigenic stimulus was provided by transfusion of A₁ blood; in the other, the source of the antigen was a fetus in utero. Because of the presence of immune anti-A₁ isoagglutinins in these patients' sera, only A₂ donors were used in subsequent transfusions, with satisfactory results. These workers presented evidence which indicated that had A₁ donors been used, hemolytic reactions most likely would have resulted. However, the absence of reports in recent literature indicates that subgroups A₁ and A₂ are not as important in transfusion as formerly believed.

Further, Wiener and associates state that in selecting donors for subgroups of A and AB, it is probably safe to disregard the subgroups except in the rare cases where the patient's serum contains an irregular isoagglutinin anti-A₁ or anti-A₂. Where the irregular isoagglutinin is present, only donors of the homologous group should be used. In this connection, the author cites a case* of a recipient of the A₂B class who had a severe reaction following donation of A₁B blood. Later, transfusion with A₂B blood resulted in no reaction. This probably corresponds with the irregular isoagglutination mentioned by Wiener.

Davidsohn⁹ maintained that the subgroups A₁, A₂, A₁B, and A₂B are significant in causing some post-transfusion reactions, but direct support of this possible mechanism was lacking until Wiener's¹⁰ report was published. Wiener analyzed a case of isoimmunization of an A₂ recipient by transfusion of A₁ blood. The anti-A₁ behaved like a "cold" agglutinin. In another case, anti-A₁

*Record of the Christian Hospital, St. Louis, Mo.

⁶Thalhimer, W.: J. A. M. A. 76: 1345, 1921.

⁷Jonsson, B.: Acta path. et microbiol. Scandinav. 13: 424, 1936.

⁸Wiener, A. S.: J. Immunol. 41: 181, 1941.

⁹Davidsohn, I.: J. A. M. A. 112: 713, 1939.

¹⁰Wiener, A. S.: Loc. cit.

antibody was considered to have developed in an A_2 woman as a result of isoimmunization by an A_1 fetus in utero. The general explanation was first presented by Levine and Stetson¹¹ as the mechanism of reactions following primary intragroup transfusions given intrapartum or postpartum.

The formation of anti-O isoagglutinin in low titer has been observed in a group A_1B patient.¹² This suggests that the production of immune anti-O isoantibodies may possibly be a factor to be considered in repeated transfusions of blood from universal donors.

Isosensitization may result not only from repeated blood transfusions but also from intramuscular injections of blood or repeated pregnancies or a combination of these, and such isosensitization may in turn be responsible for post-transfusion hemolytic reactions. The development of isoantibodies requires at least two exposures to the antigen, with an interval of several months between each exposure. The first exposure primes the individual, and subsequent exposures stimulate the production of antibodies. In some cases, a long interval is not always necessary, isosensitization resulting from continuous stimulation over a short period of time, with huge quantities of the antigen being given. Unger¹³ cites the case of an Rh-negative male patient who received 5,500 c.c. of Rh-negative blood, and then, because the patient was moribund and no more Rh-negative blood was available, he was given 54,500 c.c. of Rh-positive blood within a period of 7 days. Rh sensitization tests were negative until the one hundred eighth pint of blood was to have been administered, when it was found that the patient had developed univalent Rh_0 antibodies.

The Rh Factor

It was formerly believed that dangerous transfusion reactions could not occur if both donor and recipient belonged to the same Landsteiner blood group. Between 1921 and 1939, with the increasing use of blood transfusion, authenticated reports of hemolytic reactions began to appear in the literature. These hemolytic reactions occurred in spite of the fact that all blood grouping tests, including the cross-matching test, showed donor and recipient of the same group and apparently compatible. Apparently Unger¹⁴ reported the first case of intragroup incompatibility. Although both patient and donor belonged to group O, on cross-matching he observed some clumping of the cells. When much blood was transfused, the patient developed chills and fever. Unger believed this reaction was hemolytic in nature. He postulated the existence of two different antibodies, the anti-A anti-B, which he called the "major" agglutinins, and another antibody which he called the "minor" agglutinin. His technic for cross-matching suspended the cells in serum rather than in saline; he therefore inadvertently, even at that early date, was carrying out the conglutination test. Possibly he was detecting the Rh_0 univalent antibody, although he did not identify it.

In most of the subsequently reported cases, no explanation of reactions could be found. In a few instances, the patient's blood serum was found to

¹¹Levine, P., and Stetson, R. E.: *J. A. M. A.* 113: 126, 1939.

¹²Wiener, A. S., Oremland, B. H., Hyman, M. A., Samwick, A. A.: *Am. J. Clin. Path.* 11: 102, 1941.

¹³Unger, Lester J.: *Am. J. Clin. Path.* 24: 275, 1954.

¹⁴Unger, L. J.: *J. A. M. A.* 70: 9, 1921.

contain *irregular isoagglutinins* and *isohemolysins*. The irregular isoantibodies acted on the blood of the donor used in the transfusion, as well as on certain other human bloods, independently of the blood groups. Among the most carefully studied instances of this sort were those reported by Unger,¹ Zacho,² Culbertson and Ratcliffe,³ Neter,⁴ and Levine and Stetson.⁵ No attempt, however, was made to correlate these cases and the subject remained in this stage until Landsteiner and Wiener reported their discovery of the Rh factor and explained its role in the reactions in retransfusions.

Landsteiner and Wiener in 1937 detected the presence of M-like agglutinogens in the blood of rhesus monkeys and they found that the injection of the blood of rhesus monkeys into rabbits stimulated the production of anti-M agglutinins. Previously, Schiff found that the injection of sheep blood into rabbits caused the formation of anti-A agglutinins and hemolysins. Landsteiner and Wiener continued their research in the hope that by the use of animal blood instead of human blood for preparing antisera they might discover new factors in human blood. They did find that some of their antisera for rhesus monkey blood contained an agglutinin reacting with the blood cells of 85 per cent of the Caucasoid population, quite independently of the blood groups or M, N, and P factors. They designated this factor *Rh* to indicate the manner in which it was first discovered,⁶ using the first two letters of the word rhesus. They did not immediately report their findings because they wished to do further work on their antisera. In 1939 Wiener and Peters found three cases of hemolysis following transfusions of blood of the homologous groups, which were proved to be due to isoimmunization to the rhesus factor. Thus, this new blood factor became clinically very important,⁷ and the cases were reported. Wiener stated in his Alvarenga Prize Lecture⁸ that this was the reason why the Rh factor, found by Landsteiner and Wiener in 1937, was first announced in January, 1940.

Briefly, the blood cells of 85 per cent of all Caucasoid individuals are agglutinated by anti-rhesus (anti-Rh₀) sera. These cells contain the Rh agglutinin identified by the factor designated as **Rh₀**. These individuals were called Rh positive, while the remaining 15 per cent were called Rh negative. These Rh-negative blood cells do not possess the **Rh₀** factor and therefore are not agglutinated by anti-**Rh₀** sera. Since 1940, other Rh factors have been found, **rh'**, **rh''**, **rh^w**, and the variant **Rh₀**, along with other factors. See discussion on pages 1034 ff.

The Rh Factor in Retransfusions

In connection with blood transfusion, Wiener demonstrated that the Rh agglutinin is antigenic to man. Thus, Rh-negative individuals may become sensitized to the **Rh₀** factor as a result of the transfusion of Rh-positive blood, and then cannot again safely receive Rh-positive blood. Rh-negative indi-

¹Zacho, A.: Ztschr. f. Rassenphysiol. 8: 1, 1936.

²Culbertson, C. G., and Ratcliffe, A. W.: Am. J. M. Sc. 192: 471, 1936.

³Neter, E. L.: J. Immunol, 30: 255, 1936.

⁴Levine, P., and Stetson, R. E.: J. A. M. A. 113: 126, 1939.

⁵Landsteiner, K., and Wiener, A. S.: Proc. Soc. Exper. Biol. & Med. 43: 223, 1940.

⁶Wiener, A. S., and Peters, H. R.: Ann. Int. Med. 13: 2396, 1940.

⁷Wiener, A. S.: Alvarenga Prize Lecture V (Award XL), the College of Physicians of Philadelphia. Delivered before the College in joint meeting with the Philadelphia County Medical Society, Oct. 3, 1945. Reprinted in the Lab. Digest 10: 1, June, 1946.

viduals, except in dire emergencies, should be transfused only with Rh-negative blood, and then only if no Rh antibodies are present in the donor's serum. The rules for the Rh factor as applied to the operation of a blood bank are described by Unger.⁹

It is much safer to transfuse all Rh-negative individuals with group O Rh-negative blood only, or with Rh-negative blood compatible with their own blood groups. In respect to the transfusing of females from birth to the climacteric, if they are Rh negative and are transfused with Rh-positive blood, they may develop Rh antibodies. These qualities may persist for life, so that if another transfusion is required and Rh-positive blood is used, severe transfusion reactions may occur. Particularly the fact must be borne in mind that these Rh-negative females, who have been transfused at some time in their early life with Rh-positive blood, are isoimmunized to the Rh factor; so that when undertaking marriage and upon becoming pregnant, if the fetus is Rh positive they will most certainly have severe intragroup transfusion reactions if transfused with Rh-positive blood. If the fetus is Rh positive their chances of bearing normal infants are endangered.

The Rh Factor in Primary Transfusions of Pregnant Women

The Rh types of blood are hereditarily determined,¹⁰ the **Rh**₀ factor being transmitted as a single Mendelian dominant by a pair of allelic genes, *R* and *r* (*R* determines Rh-positive; *r* determines Rh-negative). Since every individual possesses a pair of genes from every series of allelic genes, one being derived from the mother, and the other from the father, there are three genotypes possible. Rh-negative individuals belong to genotype *rr* and are always homozygous; Rh-positive individuals may be either homozygous (genotype *RR*) or heterozygous (genotype *Rr*). Therefore, two Rh-negative parents can have only Rh-negative children. If one parent is Rh negative, and the other is Rh positive, the children will all be Rh positive if the Rh-positive parent is homozygous. If the Rh-positive parent is heterozygous, there is equal chance that the children may be Rh positive or Rh negative; or as it is usually stated, one-half of the children will be Rh positive and one-half will be Rh negative. When both parents are Rh positive, all the children will be Rh positive, except when the parents are both heterozygous, in which case one-fourth of the children will be Rh negative.¹¹

Rh-negative individuals may become sensitized to Rh antigen in one of two ways: (1) sensitization may result from a transfusion of Rh-positive blood, or (2) in women, sensitization may result from pregnancy with an Rh-positive fetus. Sensitization by either of these methods may result in a serious hemolytic reaction if transfusion with Rh-positive blood is given. Therefore, all Rh-negative women who are to be transfused must be given only Rh-negative blood.

In 1940 Levine and his co-workers¹² noted that sera of patients who had had repeated miscarriages or abortions contained atypical isoagglutinins related to the Rh factor. In 1941 Katzin found that the serum of the patient previously

⁹Unger, L. J., Weinberg, M., and Lefkon, M.: *Am. J. Clin. Path.* **16**: 498, 1946.

¹⁰Landsteiner, K., and Wiener, A. S.: *J. Exper. Med.* **74**: 309, 1941.

¹¹Wiener, A. S.: *An Rh—Hr Syllabus*, New York, 1954, Grune & Stratton, p. 22.

¹²(a) Levine, P., and Katzin, E. M.: *Proc. Soc. Exper. Biol. & Med.* **45**: 343, 1940. (b) Levine, P., Katzin, E. M., and Burnham, L.: *ibid.*, **45**: 346, 1940.

described by Levine and Stetson¹³ agglutinated the same cells as the newly discovered antirhesus rabbit serum. Levine, Katzin, and Burnham¹⁴ called attention to the fact that a diagnosis of erythroblastosis fetalis had been made on some of the dead fetuses which came from the union of Rh-positive and Rh-negative people. Vogel¹⁵ noted that the majority of the mothers had Rh-negative blood. Burnham¹⁶ and Levine¹⁷ further called attention to the relationship of the Rh factor to erythroblastosis. The doctrine that Rh-positive fetal cells entering the maternal blood stimulate the formation of anti-Rh agglutinins is now accepted. Such agglutinins pass back to the fetus and then clump and destroy the fetal erythrocytes.

An excellent description of what takes place in the placenta itself was given by Pieri and Schwartz.¹⁸ They stated that the medium for the interchange of gases, water, and nourishment between mother and baby is the fragile wall of the placental villus, a trophoblastic projection of the chorion frondosum itself. Through an arteriole, each villus is supplied with fetal blood by a branch from one of the umbilical arteries, while a corresponding venule eventually returns the blood to the fetal circulation. The delicate placental villi are bathed in pools of maternal blood, in the intervillous spaces. The maternal blood is carried to these spaces by minute branches of the uterine artery, which are usually found in relation to the decidual septa, while corresponding veins return it eventually to branches of the uterine vein. Constant circulation is assisted by the uterine contractions which occur at intervals throughout every pregnancy. Normally, the blood of the child circulates inside the villus, while the maternal blood circulates outside this delicate structure. The relationship is so close that osmosis readily takes place through the villus wall. It has been estimated that if all the villi of a placenta at full term were placed end to end their combined length would exceed eleven miles.

That the placenta is extremely susceptible to the effects of tumors, trauma, and disease has long been known. This is not a surprising observation, in view of the complex structure and function of this organ, and of the fact that the more complex an organ the more delicate and vulnerable to injury it becomes.¹⁹ Careful scrutiny of the placenta²⁰ will reveal in nearly every instance the unmistakable evidences of one or many areas of old or recent pathologic alteration. Such scars, varying in size from pinhead to several cubic millimeters, have been designated by a confusion of terms, such as infarction, hepatization, placentitis, necroses, apoplexy, etc. Actually, each such scar represents merely the evidence of a previous placental injury. Since the most delicate portion of the placenta is the villus itself, this structure is the most susceptible to injury, either as a result of apoplexy, or of hemorrhagic infarction, or of direct trauma. Whatever

¹³Levine, P., and Stetson, R.: *J. A. M. A.* **113**: 126, 1939.

¹⁴Levine, P.: Katzin, E. M., and Burnham, L.: *J. A. M. A.* **116**: 825, 1941.

¹⁵(a) Levine, P., Vogel, P., Katzin, E., and Burnham, L.: *Science* **94**: 371, 1941. (b) Levine, P., Burnham, L., Katzin, E., and Vogel, P.: *Am. J. Obst. & Gynec.* **42**: 925, 1941.

¹⁶Burnham, L.: *Am. J. Obst. & Gynec.* **42**: 389, 1941.

¹⁷(a) Levine, P.: *Am. J. Obst. & Gynec.* **42**: 165, 1941. (b) Levine, P.: *Am. J. Clin. Path.* **11**: 898, 1941.

¹⁸Pieri, R. J., and Schwartz, R. C.: *New York J. Med.* **46**: 387, 1946.

¹⁹MacCallum, W. G.: *Textbook of Pathology*, Philadelphia, 1919, W. B. Saunders Co., p. 25.

²⁰DeLee, L. B.: *Principles & Practices of Obstetrics*, ed. 7, Philadelphia 1938, W. B. Saunders Co., p. 635.

the causative factor, any violent break in the continuity of the vessels of one or more villi will result in hemorrhage of fetal blood cells into the maternal circulation. From here the fetal blood elements are carried into the uterine veins and

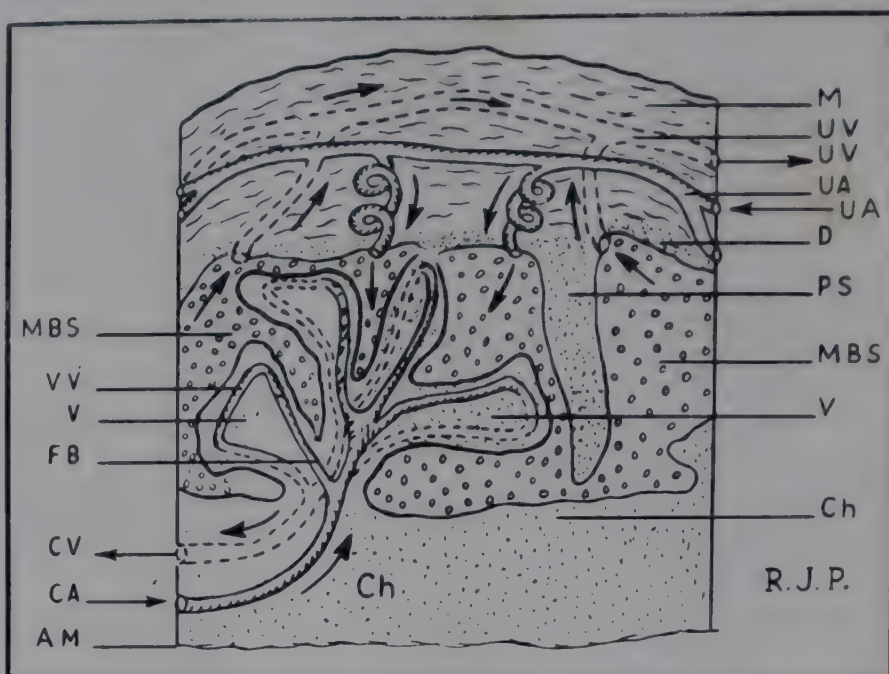


Fig. 242.—Diagrammatic section through a human placenta. *M*, Uterine muscle; *UV*, branch of uterine vein; *UA*, branch of uterine artery; *D*, decidua; *PS*, decidual septum; *MBS*, maternal blood sinus; *V*, villus; *Ch*, chorion frondosum; *AM*, amnion; *CA*, branch of umbilical artery; *CV*, branch of umbilical vein; *FB*, villus branch of umbilical artery; *VV*, vein withdrawing blood from villus. (From Pieri and Schwartz: New York J. Med. 46: 387, 1946, by permission of the authors and publisher.)

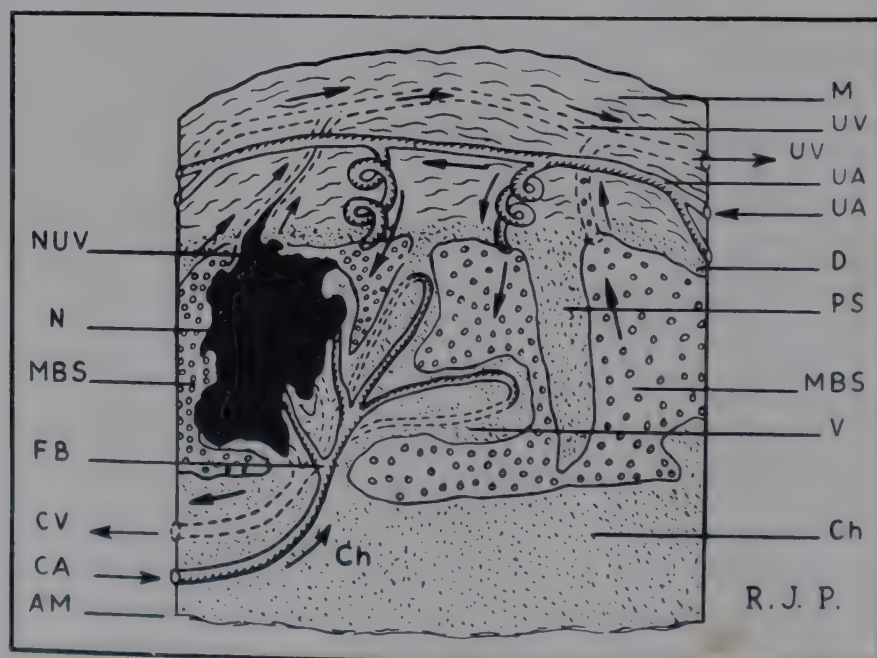


Fig. 243.—Diagrammatic illustration of rupture of placental villus. *N*, Fetal blood from villus vessels pouring into maternal blood sinus, *MBS*. Fetal blood enters maternal uterine vein at *NUV*, to gain access to general circulation of the mother (see text). (From Pieri and Schwartz: New York J. Med. 46: 387, 1946, by permission of the authors and publisher.)

thence into the general circulation of the mother. Such a hemorrhage may be minute and apparently insignificant, or of considerable size.²¹ Either may produce most serious sequelae. Now, if the fetus' cells contain the Rh antigen (Rh positive), while those of the mother are without the antigen (Rh nega-

²¹Wiener, A. S.: Am. J. Obst. & Gynec. 56: 717, 1948.

tive), a variable degree of isoimmunization may result. The Rh-positive cells of the fetus stimulate within the Rh-negative mother formation of the specific Rh antibody (anti-Rh agglutinin).

If the mother now needs a transfusion, and Rh-positive donor blood is used, there follow hemolysis and agglutination of the donor's cells with resulting renal damage or even death, from the action of the isoantibodies (anti-Rh) on the incoming Rh-positive cells. Therefore, in all primary transfusions of Rh-negative pregnant or post-partum women use Rh-negative donors.

The Rh Factor in Erythroblastosis Fetalis

The Rh antibody, formed in the blood serum of the Rh-negative mother by virtue of the presence of an Rh-positive fetus (see above) is readily transmissible, as are many toxins and antitoxins, through the placental filter (the walls of the villi) into the circulation of the infant in utero. It is the action of this antibody upon the Rh-positive cells of the baby which produces the disease entity which in about 90 per cent of the cases is recognized as isohemolytic disease or erythroblastosis fetalis. In most of the remaining 10 per cent the disease apparently depends upon similar genetically related antigens (such as those designated as Hr or other blood group antigens), present in the baby's cells but absent in those of the mother.

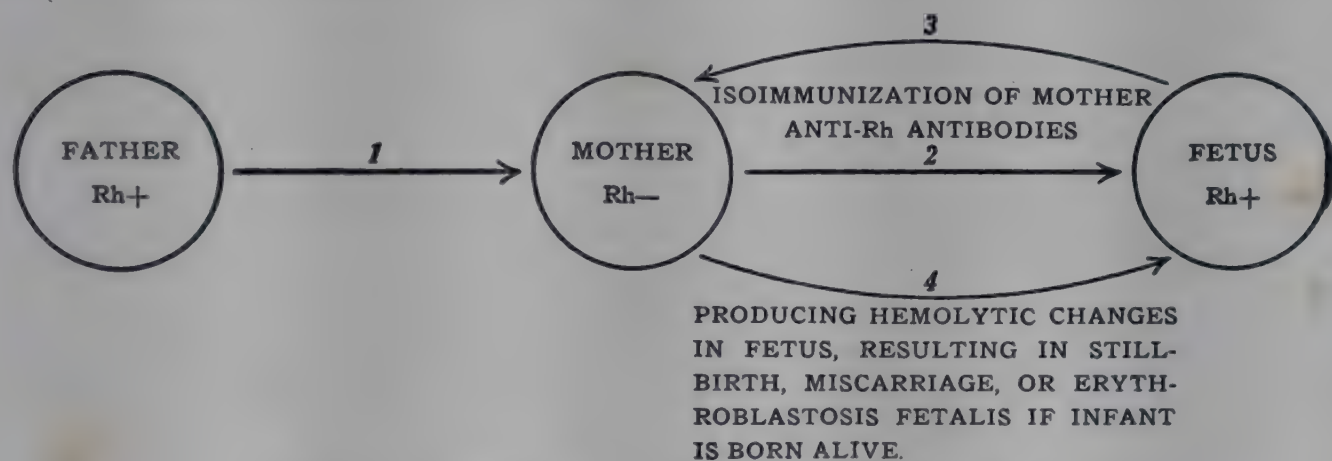


Fig. 244.—Diagram of the Rh phenomenon in pregnancy.

Fig. 244 illustrates what takes place in pregnancy when an Rh-negative woman is mated to an Rh-positive man. The Rh-positive fetus stimulates formation of Rh antibodies in the mother (isoimmunization). Anti-Rh agglutinins from the mother pass through an imperfect placental barrier, producing hemolytic changes in the fetus. This will result in miscarriage, stillbirth, or erythroblastosis fetalis if the infant is born alive.

While the mating of Rh-negative women with Rh-positive men occurs in one of ten marriages, congenital hemolytic disease affects only one in about two hundred and fifty newborn. This suggests that only about one in twenty-five Rh-negative women becomes sensitized when bearing an Rh-positive fetus, just as in sensitization by blood transfusion. The first-born is rarely affected, because it takes at least one pregnancy, and sometimes more, before a sufficient degree of sensitization develops. In most of the rare cases where the first-born was affected, a careful history has revealed that the women had previously received a transfusion of Rh-positive blood. This serves, according to Wiener,

to emphasize the importance of performing Rh tests before giving blood transfusions to women in the child-bearing age, as well as to young girls, because a transfusion of the wrong Rh type blood could ruin a woman's chances of having a normal child. Refer to pages 1038, 1043, and 1060 for a discussion of univalent antibodies.

Summary of Facts Relative to the Rh Factor

1. In Retransfusions.—

If the recipient is Rh negative and the donor is Rh positive, the Rh agglutinin in the donor's blood cells acts as an antigen and stimulates the formation of anti-Rh agglutinins in the patient's serum. The patient is now isoimmunized to the Rh factor.

If this patient is again transfused with Rh-positive blood cells, the Rh antibodies, formed as a result of the first transfusion, agglutinate or hemolyze the incoming donor cells, resulting in a transfusion reaction or death.

Therefore, since Rh-negative blood has no **Rh_o** factor, always use Rh-negative donor blood in retransfusions of Rh-negative patients.

2. In Primary Transfusions of Pregnant and Post-partum Women.—

If the mother is Rh negative and the father is Rh positive, the fetus will most likely be Rh positive. Through an imperfect placenta, the Rh-positive fetal blood seeps into the mother's circulation, causing the mother to form Rh antibodies (agglutinins) in her blood serum. She is now isoimmunized to the Rh factor.

If this isoimmunized woman were given a transfusion with Rh-positive blood, the Rh antibodies now present in her blood plasma react with the incoming donor blood cells and agglutinate them or hemolyze them, or both, resulting in a transfusion reaction or death.

Therefore, since Rh-negative blood cells contain no Rh agglutinin, always transfuse Rh-negative pregnant or post-partum women with Rh-negative blood.

3. In Erythroblastosis Fetalis.—

If the mother is Rh negative and the father is Rh positive, the fetus will frequently be Rh positive. Through an imperfect placenta, the Rh-positive blood seeps into the maternal circulation, stimulating the formation of Rh antibodies, and isoimmunizing the mother. Her blood serum now contains Rh antibodies. Through imperfections in the placenta,* some of the mother's blood, containing Rh agglutinins, now re-enters the fetal circulation, and agglutinates the Rh-positive cells. They also attack the hematopoietic system. As a result, the fetus is either expelled as an abortion, is macerated, is still-born, or, if born alive, develops erythroblastosis fetalis.

If the infant is transfused with Rh-positive blood cells, the Rh antibodies in the infant's blood (derived from the mother) react with the incoming Rh-positive donor blood cells and a reaction ensues.

Therefore, in all pregnancies where the mother is Rh negative and the father is Rh positive, be prepared for an erythroblastotic infant, and transfuse

*See discussion of univalent antibodies, page 1038.

the infant immediately upon birth with Rh-negative donor blood. If no Rh-negative donor is available, use the *washed* red cells of the Rh-negative mother. Washing the cells removes the anti-Rh antibodies. In emergencies, however, the mother's whole blood may be used.* See pages 1075 ff. for exsanguination transfusions.

Subtypes of Rh

It was found by Wiener** that the great majority of the human antisera gave reactions identical with those of the anti-rhesus rabbit sera, and such human antisera are now designated as standard anti-Rh or anti-**Rh_o**. The differences among human antisera were soon explained by the demonstration of two additional varieties of Rh agglutinins, one giving 70 per cent positive reactions and designated as anti-**rh'**, the other giving 30 per cent positive reactions and designated anti-**rh''**. If blood specimens are tested with all three antisera, anti-**Rh_o**, anti-**rh'**, and anti-**rh''**, eight distinct types of human blood can be identified instead of two, Rh positive and Rh negative, detected with the aid of standard human anti-Rh serum or animal anti-rhesus serum alone. As Wiener^{1, 2} has shown, the three Rh antisera detect three corresponding Rh factors, **Rh_o**, **rh'**, and **rh''**, which in combination give rise to at least five Rh agglutinogens instead of one; namely, **Rh_o**, **rh'**, **rh''**, **Rh₁** (or **Rh'_o**), and **Rh₂** (or **Rh''_o**). These five agglutinogens in combination determine the eight Rh types, which are hereditarily transmitted by means of at least six allelic genes, **R^o**, **r'**, **r''**, **R¹**, **R²**, and **r**.

The Rh blood types, like the blood groups and M and N types, exhibit striking differences in distribution in various races and have important applications in anthropology.³ Wiener has studied these facts in connection with races such as Chinese, Japanese, American Indians, Filipinos, Hawaiians, and Australian aborigines. In Negroes, he found the most striking finding was the high incidence of type **Rh_o**, which was about twenty times as frequent as in whites.

THE EIGHT Rh BLOOD TYPES*

DESIGNATION OF TYPES		REACTIONS WITH SERA			GENOTYPES
		ANTI- rh'	ANTI- rh''	anti- Rh_o	
Blood Containing Rh_o	Rh_o	-	-	+	R^oR^o and R^or
	Rh₁ (Rh'_o)	+	-	+	R¹R¹ , R¹r' , R¹r , R¹R^o , R^or'
	Rh₂ (Rh''_o)	-	+	+	R²R² , R²r'' , R²r , R²R^o , R^or''
	Rh₁Rh₂ (Rh'_oRh''_o) (Rh_z)	+	+	+	R¹R² , R¹r'' , R²r'
Blood Lacking Rh_o	rh	-	-	-	rr
	rh'	+	-	-	r'r' and r'r
	rh''	-	+	-	r''r'' and r''r
	rh'rh'' (rh_y)	+	+	-	r'r''

*This table does not contain the **Rh_o** variant or genes **r^y** and **R^z**.

*Wiener, A. S., Wexler, I. B., and Brancato, G.: J. Pediat. 45: 546, 1954.
**Wiener, A. S.: The Rh-Hr Blood Types; Applications in Clinical and Legal Medicine and Anthropology, New York, 1954, Grune & Stratton.
¹Wiener, A. S.: Proc. Soc. Exper. Biol. & Med. 54: 316, 1943.
²Wiener, A. S.: Science 99: 532, 1944.
³Wiener, A. S., Sonn, E. B., and Belkin, R. B.: Proc. Soc. Exper. Biol. & Med. 54: 238, 1943.

The Hr Factor

Levine and Javert⁴ reported that they had detected in the serum of an Rh-positive mother of an erythroblastotic infant an antibody which had the property of agglutinating all Rh-negative bloods. Because of this property of the serum, the new agglutinable property detected by it was designated as Hr, and the corresponding agglutinin as anti-Hr. Levine reported that his anti-Hr serum gave about 30 per cent positive reactions and that all Rh-positive bloods not agglutinated by anti-rh' reacted with anti-Hr. On this basis he postulated that Hr and Rh were allelic, without further clarifying his hypothesis. He also asserted that Hr incompatibility must be considered as a possibility whenever an Rh-positive mother has an Rh-negative child, and that all type Rh₁Rh₂ individuals are Hr negative. These two statements, according to Wiener's observations and others, have been contradicted by subsequent findings. For instance, in 1943, Race and Taylor⁵ also detected in the serum of an Rh-positive mother of an erythroblastotic infant an agglutinin which reacted with all Rh-negative bloods, but differed from Levine and Javert's in that it gave 80 per cent positive reactions. As already pointed out by Wiener and associates,⁶ Race and Taylor's serum detected the same blood property as Levine and Javert's serum, the lower percentage of positive reactions obtained by Levine being due to the use of a weaker antiserum which missed bloods heterozygous for the Hr factor. As postulated by Race and associates,⁷ Hr is a blood factor present in the agglutinogens determined by genes R^2 , r'' , R^0 , and r , but absent from the agglutinogens determined by genes R^1 and r' and r^y and R^z . Wiener postulates that a single dose of a gene positive for Hr determines a weaker reaction with anti-Hr than a double dose of Hr-positive genes.^{5, 6}

The main clinical application of the Hr factor is as a presumptive test for homozygosity or heterozygosity of type Rh₁ fathers in families with erythroblastotic infants.⁵ The diagnosis is only presumptive because there are five genotypes in type Rh₁, while anti-Hr serum merely subdivides type Rh₁ into two parts.⁶ Hr'-negative individuals of type Rh₁ are almost surely homozygous for Rh₁, while hr'-positive individuals of type Rh₁ are almost always heterozygous and bear gene r .

The Rh-Hr Blood Types and Their Variants

As previously stated, Landsteiner and Wiener, in 1937, were able to immunize rabbits against the blood of rhesus monkeys. When they tested the antiserum produced in the rabbits against blood cells of human beings, they found that 85 per cent of those tested reacted with the anti-rhesus serum. They then postulated that 85 per cent of human beings contain in their serum an agglutininogen similar to that found naturally in rhesus monkeys. They called the factor in the cells "**Rh**," using the first two letters of the word "rhesus." Those whose blood contained this factor were designated as Rh

⁴Levine, P., Burnham, L., Katzin, E. M., and Vogel, P.: *Am. J. Obst. & Gynec.* 42: 925, 1941.

⁵Race, R. R., and Taylor, G. L.: *Nature* 152: 300, 1943.

⁶Wiener, A. S., Davidsohn, I., and Potter, E. L.: *J. Exper. Med.* 81: 63, 1945.

⁷Race, R. R., Taylor, G. L., Cappell, D. F., and McFarlane, M. N.: *Nature* 153: 52, 1944.

positive, while those whose blood lacked the factor were called Rh negative. Later it was found that the blood serum of human beings who had been iso-immunized as a result of transfusions or of pregnancy contained the same antibody as the original anti-rhesus rabbit serum, and, in addition, two other related antibodies were found. Wiener then called the original antibody "anti-Rh₀," and the blood factor which stimulated its formation "Rh₀." The related Rh factors were designated "**rh**'" and "**rh**'," and the antisera "anti-**rh**'" and "anti-**rh**.'"

Since the **Rh**₀ factor is the most antigenic of the three, and since it causes more than 90 per cent of the cases of erythroblastosis fetalis and intragroup hemolytic transfusion reactions, it is written with a large letter *R*. The other two factors, **rh**' and **rh**'', are less antigenic, and are therefore written with the small letter *r*. Approximately 85 per cent of Caucasoids, 95 per cent of Negroids, and almost 100 per cent of Mongoloids have the Rh₀ factor in their blood.¹

The **Rh**₀ factor is found alone in 2.5 per cent of the population, and in combination with either **rh**' or **rh**'' or both in 82.5 per cent, so that 85 per cent of the population have the **Rh**₀ factor and 15 per cent lack it. The **rh**' factor is found alone in 1.0 per cent of the population, and combined with either **Rh**₀ or **rh**'' or both in approximately 70 per cent. The **rh**'' factor is found alone in only 0.5 per cent of people, but in combination with either **Rh**₀ or **rh**' or both in 30 per cent. Approximately 87 per cent of the population, therefore, are Rh positive, and 13 per cent type rh. The Rh factors do not represent separate structures on the red cell envelope, but are attributes of complex structures, the Rh agglutinogens, and each is characterized by one or more Rh factors. The **rh**^w factor, for example, occurs only in association with factor **rh**'.

By combination of the three principal Rh factors, the following Rh agglutinogens have been identified (the types are described in Table 102):

<i>Rh Agglutinogen</i>	<i>Factors Present</i>
Rh ₀	Rh ₀
Rh ₀ ' = Rh ₁	Rh ₀ and rh '
Rh ₀ '' = Rh ₂	Rh ₀ and rh ''
Rh ₀ ''' = Rh ₃ (Rh ₁ Rh ₂)	Rh ₀ , rh ', and rh ''
rh	No Rh factors
rh'	rh '
rh''	rh ''
rh _y (rh'rh'')	rh ' and rh ''

One must distinguish between "blood factors" and "agglutinogens." An agglutinogen is a definite hereditary substance of unknown chemical composition present on the red cell envelope; red cells have a number of such agglutinogens. Blood factors are attributes or properties of the structure called an agglutinogen. Each agglutinogen has multiple blood factors which are the serologic attributes by which the agglutinogen can be identified. Agglutinogens are antigenic and capable of inducing antibody formation in the serum of susceptible subjects, specific for one or more of its various blood factors; such sera are used for diagnostic purposes. The convention has been adopted

¹Wiener, A. S.: *An Rh-Hr Syllabus*, New York, 1954, Grune & Stratton.

to print symbols of blood factors and their specific antisera in bold-face type; symbols for agglutinogens and phenotypes are printed in regular type; genes and genotypes are printed in italics.

Adequate methods must be used to determine Rh factors and to identify Rh antibodies and other atypical antibodies.

Since the **Rh₀** factor is the most antigenic of the Rh-Hr factors, and therefore the one to which most human beings respond most readily, it is clinically the most important. No patient whose blood lacks the **Rh₀** factor should be transfused with blood that contains it. Thus, recipients who are type rh', type rh'', or type rh₀ must be given only Rh-negative blood, since they lack the **Rh₀** factor and can be isoimmunized against it if given blood containing **Rh₀**. On the other hand, if a donor belongs to type rh', type rh'', or type rh₀, such blood must *not* be given to Rh-negative recipients, for such recipients can produce anti-rh' or anti-rh'' antibodies, so that if the donor belongs to any of these three types, such blood should be given only to Rh-positive individuals.

The **Hr** factors are factors reciprocally related to the Rh factors, and can be found only when the corresponding Rh factor is absent. The symbol "Hr" was first suggested by Levine to indicate the relationship between Rh and Hr. Antisera for factor hr' and factor hr'' have been found, but no anti-Hr₀ serum has as yet been discovered. The hr' factor occurs in 80 per cent of the population, and the hr'' in 98 per cent, so that only 20 per cent lack the hr' factor and 2 per cent lack the hr'' factor. The Hr factors are much weaker antigens than the **Rh₀** factor. This explains the relative infrequency of transfusion reactions and cases of erythroblastosis fetalis due to the Hr factors. Nevertheless, they are important and must not be ignored.

Since the Hr factors are counterparts of the Rh factors, those who are rh' negative are always hr' positive, and those who are rh'' negative are hr'' positive,* and vice versa.

Antisera for the Rh-Hr types now available are anti-**Rh₀**, anti-rh', anti-rh'', anti-rh₀, anti-hr', and anti-hr''. In addition, a new antibody has recently been found, called "anti-hr." This is specific for a factor in the agglutinogens determined by the *R*⁺ and *r* genes, but does not increase the number of phenotypes.† (See page 1053.) There is also an anti-rh⁺ serum, which reacts with 4 to 8 per cent of all bloods. Each antiserum reacts with its specific blood factor to cause agglutination.

With the three basic anti-Rh sera, eight Rh types are distinguishable. Table 102.

Wiener selected the names Rh₀ and Rh₁ for the sake of simplicity and also to indicate that the blood factors in such bloods are usually combined to form a unit agglutinogen inherited by a corresponding gene.

If a fourth antiserum is used, namely, anti-rh⁺, types rh', Rh₀, rh₀, and Rh₁ can be subdivided into those which give negative results and those

*Rare exceptions to this rule have recently been described (Wiener, A. S., Gordon, E. B., and Cohen, A., *Ann. N. Y. Acad. Sci.*, 4: 144, 1947; Rosenfield, R., et al., *Proc. M. J. 1: 912, 1947; Wiener, A. S., *Brit. M. J.* 1: 1091, 1947).*

TABLE 102.—REACTIONS WITH ANTISERA

ANTI rh'	ANTI rh''	ANTI Rh ₀	TYPE	Rh FACTORS PRESENT
-	-	-	rh, or triple Rh negative	None
+	-	-	rh'	rh'
-	+	-	rh''	rh''
+	+	-	rh, rh'	rh', rh''
-	-	+	Rh ₀	Rh ₀
+	-	+	Rh ₀ , rh'	Rh ₀ , rh'
-	+	+	Rh ₀ , rh''	Rh ₀ , rh''
+	+	+	Rh ₀	Rh ₀ , rh', rh''

which give positive results, and the latter are named rh'', Rh'', rh''rh'' and rh''Rh₀. (Factor rh'' occurs only in bloods containing rh').

There are two main Hr antisera, anti-hr' and anti-hr''. If an Rh factor is absent, the corresponding Hr factor is present and vice versa. Exceptions to this are extremely rare. If, however, an Rh factor is present, the corresponding Hr factor may or may not be present. Thus, when the three anti-Rh sera and the two anti-Hr sera are used simultaneously, 18 Rh-Hr types are determined. If a third Hr antiserum, anti-Hr₀, were found, the number of Rh-Hr types would be increased to 27. Sensitization by hr' and hr'' factors can cause clinical complications indistinguishable from those caused by the Rh factor. Ideally, a complete Rh-Hr typing should be made of both donor and recipient in each transfusion, and only type-specific blood transfused, but at present this is not possible.

The Rh₀ factor is the variant of the Rh factor, and is called the "Rh₀ variant." It must be tested for if the standard Rh factor is found to be absent.

If all 5 testing sera are used to test unknown blood, one no longer is justified in saying merely "Rh positive" or "Rh negative." The designations of types should be made according to Table 103.

TABLE 103

ANTISERA USED					DESIGNATION OF TYPES
ANTI Rh ₀	ANTI rh'	ANTI rh''	ANTI hr'	ANTI hr''	
-	-	-	-	-	Rh ₀
-	-	-	-	+	rh'rh'
-	-	-	-	+	rh''rh''
-	-	-	+	-	rh'rh''
-	-	-	+	+	rh, rh'
-	-	-	-	+	rh, rh''
-	-	-	-	+	rh, rh'
-	-	-	-	+	rh, rh''
-	-	-	-	+	rh, rh'
-	-	-	-	+	Rh ₀
-	-	-	-	+	Rh ₀ , Rh ₀
-	-	-	-	+	Rh ₀ , rh'
-	-	-	-	+	Rh ₀ , Rh ₀
-	-	-	-	+	Rh ₀ , rh'
-	-	-	+	-	Rh ₀ , Rh ₀
-	-	-	+	+	Rh ₀ , Rh ₀
-	-	-	+	+	Rh ₀ , Rh ₀
-	-	-	+	+	Rh ₀ , Rh ₀

The Nature of the Rh-Hr Antibodies

There are two distinct varieties of Rh-Hr antibodies of any given specificity, formed as a result of isoimmunization with the Rh agglutininogen, and each reacts best in the laboratory under different test conditions.

The first variety, called "agglutinins" or "bivalent antibodies," or "complete" or "early immune" antibodies, clumps specific cells when these are suspended either in saline or in high colloid medium, or if these same cells are enzyme-treated and suspended in saline.

The second variety, called "glutinins" or "blocking" or "univalent" or "incomplete" or "hyperimmune" antibodies, does not produce visible clumping of specific cells when these are suspended in saline, but simply combines with the factor for which it is specific, and "coats" the cells or "blocks" the reaction. (Coating of the cells can be detected by the antiglobulin or Coombs test.) This variety of antibody will clump specific cells by its combined action with conglutinin normally present in serum, or by added colloidal substitutes such as acacia or gelatin (conglutination), and also when specific cells have been enzyme-treated and then suspended in saline and then followed by the antiglobulin test (Unger test).

There is evidence that there may be a third variety of antibody which "coats" cells but lacks the capacity to clump them when they are suspended either in saline or in high colloid medium, but this coating may be detected by the antiglobulin method.

Wiener¹ described the difference between the two varieties of antibodies and their reaction in vitro as follows:

Anti-Rh agglutinins (bivalent) + Rh-positive cells = Rh agglutination.

Anti-Rh glutinins (univalent, or blocking antibodies) + Rh-positive cells + X protein = Rh conglutination. The third component listed here, X protein, is a normal component of blood serum. It is adsorbed onto the specifically sensitized red cells, causing them to stick together. This X protein is distinct from complement. It resists heating at 60° C. and is a colloidal constituent or conglutinin in the plasma, a large molecular complex of albumin, globulin, fibrinogen, and phospholipid. X protein, although heat-resistant, dissociates readily into its constituent smaller molecules of albumin and globulin upon dilution of the plasma with water. Use of saline, to suspend cells or to dilute serum, either partially or completely dissociates the X protein. Thus, in order to carry out conglutination tests, any crystalline solution must be strictly avoided.

Univalent or blocking antibodies are very important. They tend to appear usually late in the course of immunization, are relatively heat-resistant, and readily pass through the intact placenta (Wiener).

Bivalent antibodies are usually produced early in the course of immunization, are relatively thermolabile, and are effectively held back by the intact placental barrier.

Other Blood Systems

The Lewis System.—Two related Lewis factors have been found, designated **Le^a** and **Le^b**, respectively. Adults who are **Le^a**-positive almost invari-

¹J. Lab. & Clin. Med. 30: 662, 1945.

ably are nonsecretors of the A-B-O group substances, while **Le^a**-negative persons are secretors (Grubb*). Since great difficulty has been encountered in obtaining potent and specific reagents for detection of the Lewis factors, many aspects of this system are still unclear.¹

Kell-Cellano Types (K-k).—Antibodies for a blood property present in approximately 10 per cent of Caucasoids have been found in occasional sera from mothers of erythroblastotic babies, and also in sera of persons who have had hemolytic transfusion reactions.² The symbol "**K**" was selected for this factor after the patient, Kell, in whose serum the antibody was first detected.

More recently an antibody was encountered in the serum of a mother of an erythroblastotic baby which gives reactions reciprocally related to the Kell factor. This factor was designated "Cellano," after the patient.³ It has an incidence of 99.8 per cent among Caucasoids. It has been assigned the symbol "**k**" to indicate its reciprocal relationship to the Kell factor. When tests are made with both anti-**K** and anti-**k** sera, three types can be differentiated: type KK in 0.2 per cent; type Kk in 8.6 per cent; and type kk in 91.2 per cent.

Most of the anti-**K** sera must be used with the agglutination and antiglobulin methods. Limited quantities of anti-**K** serum are commercially available.

Duffy or Fy^a Factor.—The so-called *Duffy*, or **Fy^a** factor has been the occasional cause of hemolysis in isosensitized patients. These reactions have been serious, and therefore the factor is important. They are demonstrable only by the antiglobulin technic.

Blood Factors S and s, of the M-N-S Group.—For a long time, the M-N types were considered to constitute a simple system of only three types, M, N, and MN, but with the development of new methods for detecting immune iso-antibodies, it was demonstrated that the M-N system is far more complicated than at first believed.

In 1947, Walsh and Montgomery⁴ found in the serum of a mother of an erythroblastotic baby not only an Rh antibody, but also a second antibody. This gave reactions related to the M-N system. The new agglutinin was designated by the symbol "**S**" and the corresponding antibody as "anti-**S**."

In 1951, Levine and associates⁵ detected an antibody in the serum of a mother of an erythroblastotic baby which gave reactions reciprocally related to the agglutinin S. The new agglutinin was designated "**s**," and the corresponding antibody "anti-**s**."

Discovery of anti-**s** serum has increased the interest and importance of the M-N-S system, because when all four antisera are available, it becomes possible to differentiate as many as 9 phenotypes corresponding to the 10 theoretically possible genotypes.

Only a limited quantity of the antisera is available at present.

*Grubb, R.: *Nature* **162**: 933, 1948.

¹Wiener, A. S.: *Lab. Digest* **18**: Nov., 1954.

²Coombs, R. R. A., Mourant, A. E., and Race, R. R.: *Lancet* **1**: 264, 1946; Wiener, A. S., and Sonn-Gordon, E. B.: *Rev. hémat.* **2**: 3, 1947.

³Levine, P., Wigod, M., Backer, A. M., and Ponder, R.: *Blood* **4**: 869, 1949.

⁴Walsh and Montgomery: *Nature* **160**: 504, 1947.

⁵Levine et al.: *Proc. Soc. Exper. Biol. & Med.* **78**: 218, 1951.

Blood Factor U.—In 1953, Wiener, Unger, and Gordon⁶ found in the serum of a Negroid woman an abnormal antibody which strongly agglutinated the cells of the two donors whose blood had been used in transfusing her.

This was a case of a fatal hemolytic reaction in a woman who entered the hospital with a diagnosis of bleeding peptic ulcer. She was given a transfusion, but this was discontinued after 100 c.c. of blood had been administered, because of a reaction consisting of chills and fever. Another blood transfusion was carried out a week later. The patient died shortly thereafter.

The antibody found in the serum was of high titer and avidity. At body temperature, the titer was approximately 400 units, and at refrigerator temperature only 40 units. This antibody had the property of an immune iso-antibody in contradistinction to naturally occurring antibodies such as anti-**A**, anti-**B**, and anti-**P**. It was active by the ordinary agglutination method in saline medium. The blood factor was not affected by proteolytic enzymes.

Tests made on a short series of blood specimens showed them all incompatible with the patient's serum; therefore the factor in question has a high frequency in the population. It apparently is not related to any known blood factor, and has been given the name "**U**."

No Caucasoids out of 690 tested have been found to give a negative reaction to the antisera, and out of 425 Negroids, only four were negatively reacting.

* * * * *

With the increase in the number of transfusions and the discovery of so many blood factors not heretofore known, it has become increasingly evident that blood grouping is a highly specialized field, and these delicate tests must be carried out with precision by specially trained persons if fatal hemolytic reactions are to be avoided.

Summary of the Blood Groups in Man and Their Role in Transfusion

Most of the newly described factors found in blood have been discovered as a result of a hemolytic transfusion reaction or the birth of an erythroblastic infant, which could not be explained on the basis of Rh_0 sensitization. Application of the tests for univalent antibodies to a study of such cases may reveal the presence of an unusual antibody in the patient's serum, and thus most of the newer factors are potentially important clinically. However, it is not necessary to test all recipients of blood transfusions or every pregnant woman for all of these factors. Observations have shown that while the majority of Rh-negative individuals injected with Rh-positive blood eventually become sensitized, this is not true for other blood factors. For example, injections of hr' -positive blood into hr' -negative individuals experimentally seldom stimulate production of specific antibodies; this indicates that hr' is far less antigenic than the Rh_0 factor. The same appears to be true for blood factors rh' , rh'' , rh^w , hr'' , **F**, **K**, **S**, etc. It is therefore adequate to test prospective recipients of blood transfusion for the A-B-O groups and the Rh_0 factor and to rely on proper cross-matching tests and tests for sensitization to detect incom-

⁶Wiener, Unger and Gordon.: J.A.M.A. 153: 1444, 1953.

TABLE 104.—BLOOD GROUPS IN MAN: ROLE IN BLOOD TRANSFUSION¹

SYSTEM	BLOOD FACTORS	NUMBER OF IDENTIFIABLE TYPES		ISOANTIBODIES		ROLE IN CAUSING TRANSFUSION HEMOLYSIS	
		NATURAL	IMMUNE	Common	Most important	Common	Most important
A-B-O	A, B, C, O, and variants such as A ₁ , A ₂ , A ₃ , A _{1,2} and A ₃ , etc.	6 major groups and subgroups—O, A ₁ , A ₂ , B, A ₁ B, A ₂ B; also rarer subgroups A ₃ , A ₃ B, etc.	Regularly present except during neonatal period	Common	Has caused hemolysis in rare cases	Most frequent cause of intragroup transfusion hemolysis	Most frequent cause of intragroup transfusion hemolysis
M-N-S	M, N, S, s, Hu (Hunter), He (Henshaw), and variants such as M ₂ , N ₂ , etc.	6 major types using the more readily available sera and anti-M, anti-N, and anti-S	Rare	Rare	Has caused hemolysis in rare cases	Most frequent cause of intragroup transfusion hemolysis	Most frequent cause of intragroup transfusion hemolysis
Rh-Hr	Rh ₀ , rh', rh'', rh ^w , hr', hr'', and variants such as Rh ₀	28 major types, but of these only 9 have frequencies greater than 1%	A few questionable cases reported	Common	Most frequent cause of intragroup transfusion hemolysis	Most frequent cause of intragroup transfusion hemolysis	Most frequent cause of intragroup transfusion hemolysis
P	P and variants	2 major types	Infrequent	Very rare	No cases reported	No cases reported	No cases reported
K-k	K and k	3 types	None reported	Occasional	Rare cause of hemolysis in isosensitized patients	Rare cause of hemolysis in isosensitized patients	Rare cause of hemolysis in isosensitized patients
Lutheran	Lu	2 types	None reported	1 case	Very rare cause of hemolysis in isosensitized patients	Very rare cause of hemolysis in isosensitized patients	Very rare cause of hemolysis in isosensitized patients
Duffy (Fy)	F and f	2 major types, because anti-f serum is not available	None reported	Several cases	Rare cause of hemolysis	Rare cause of hemolysis	Rare cause of hemolysis
Lewis (Le)	Lea and Leb	3 types (?)	Infrequent	None found	Plays no role in transfusion hemolysis. Lea factor reciprocally related to secretor trait	Plays no role in transfusion hemolysis. Lea factor reciprocally related to secretor trait	Plays no role in transfusion hemolysis. Lea factor reciprocally related to secretor trait
Kidd (Jk)	J and j	2 major types, because anti-j serum is not available	None reported	Few cases	Rare cause of hemolysis	Rare cause of hemolysis	Rare cause of hemolysis
“Private” blood types*							
Rare blood factors	Leavy, Gr (Graydon), Mia (Miltenberger), Becker, Bea (Berrens), Ca (Cavaliere), Wra	Positively reacting bloods are very rare	None reported	Very rare	Very rare cause of hemolysis	Very rare cause of hemolysis	Very rare cause of hemolysis
Factors with almost universal distribution†	Tja and U	Negatively reacting bloods are very rare	None reported	1 case anti-U; 8 cases anti-Tja	Very rare cause of transfusion hemolysis	Very rare cause of transfusion hemolysis	Very rare cause of transfusion hemolysis

*In the case of the rare blood factors, other individuals possessing such factors are most easily found by testing relatives of the proband, the person in whose blood the factor was first found. Similarly, individuals, lacking factors of almost universal distribution are to be found most readily among relatives of the proband. The hereditary nature of the blood factors, in general, is most noticeable in the case of very rare blood types, and Levine has, therefore, designated them as “private” blood types to indicate their restriction to certain rare families.

†Factor k of the K-k blood types could also be included under this classification.

¹From Wiener: New York J. Med. 54: 3071, 1954.

patibilities with regard to the other human blood factors. Such incompatibilities are rare, and will be found only in individuals who have had previous transfusions or pregnancies which could have sensitized them.

Some of these factors are demonstrable only by the antiglobulin method. Table 104 is a résumé of the blood groups and their role in transfusion.

Rh-Hr Glossary*

Antigen: Any substance which when introduced into the body, usually by a parenteral route, stimulates the formation of specific reacting substances.

Antibodies: Specifically reacting substances, believed to be globulins in chemical structure, produced by the body in response to the introduction of an antigen.

Red Cell Antibodies: Substances occurring in blood serum which react with their specific antigen or agglutinin, which are found on the surface of red cells. Some antibodies clump cells, some coat cells, some lyse cells, etc.

Agglutinin: A substance on the surface of red blood cells which, in combination with its specific antibody (agglutinin), results in clumping of the cells. Agglutinogens are named for the most important blood factor or factors which characterize them. For example, agglutinin A is named for blood factor **A**, although it shares factor **C** with agglutinin B. (Agglutinin A also has a factor **F_A**, which it shares with sheep cells.)

Anti-rhesus serum: In the narrow sense, antiserum prepared in rabbits, guinea pigs, goats, and other animals in response to the injection of blood of rhesus monkeys. Thus, the term applies to sera like the original experimental serum of Landsteiner and Wiener, which clumps the bloods of 85% of all Caucasoids. In the broad sense, the term has been applied to include also all related Rh and Hr antisera of human origin.

Isoimmunization; isosensitization: Production of antibodies by an individual of a species in response to stimulation by an antigen derived from a different member of the same species. (See pages 1024 ff.)

Rhesus factors; Rh factors; Rh agglutinogens; Rh antigens: Structures on the surface of red blood cells responsible for the reactions with Rh antisera. Study of anti-Rh sera of human origin show that there are a variety of factors related to the original rhesus factor of Landsteiner and Wiener. The one corresponding to the original factor is designated **Rh₀**. Reciprocally related factors are termed **Hr factors**. There are four Rh factors: **Rh₀**, **rh'**, **rh''**, and **rh^w**. Three Hr factors have been found: **hr'**, **hr''**, and **hr**. There are also minor variants of the Rh-Hr factors.

Rhesus antibodies, Rh antibodies: The specific substances responsible for the reactions produced by anti-rhesus sera. The anti-rhesus (anti-Rh) sera prepared by immunizing animals with rhesus blood give parallel reactions with human bloods. The anti-rhesus (anti-Rh) sera of human origin are of 7 principal varieties. (1) Rh antibodies: anti-**Rh₀**, anti-**rh'**, anti-**rh''**, and anti-**rh^w**; (2) Hr antibodies: anti-**hr'**, anti-**hr''**, and anti-**hr**.

Rh₀ factor; standard Rh factor: This is the original Rh blood factor which corresponds to the original rhesus factor of Landsteiner and Wiener, and is present in the blood of approximately 85% of Caucasoids, 95% of Negroids, and almost 100% of Mongoloids. It is the most antigenic of the Rh-Hr factors, and therefore the most important clinically. More than 90% of cases of erythroblastosis fetalis and intra-group hemolytic transfusion reactions are due to this factor. When one speaks of the Rh factor, it usually is assumed that the **Rh₀** factor is meant. The symbol for the factor **Rh₀** is written with a large "R" to indicate that this is by far the most antigenic of the factors.

*Wiener, A. S.: Lab. Digest 12: 12, 1949; 14: 6, 1950; Wiener, A. S.: An Rh-Hr Syllabus, New York, 1954, Grune & Stratton.

Anti-Rh₀ serum; standard anti-Rh serum: Serum specific for the Rh₀ factor. Only anti-Rh₀ serum of human origin is used for diagnostic purposes, since anti-rhesus immune animal serum clumps all blood specimens from newborn infants.

Rh testing: Classifying human blood by tests with anti-Rh₀ serum alone. This divides all bloods into two types, Rh positive and Rh negative, or, strictly speaking, Rh₀ positive and Rh₀ negative.

TABLE 105.—SCHEME OF THE EIGHT Rh BLOOD TYPES

BLOOD CONTAINING FACTOR Rh ₀ (Rh ₀ POSITIVE)				BLOOD NOT CONTAINING FACTOR Rh ₀ (Rh ₀ NEGATIVE)			
DESIGNATION OF TYPES*	REACTIONS WITH SERA			DESIGNATION OF TYPES*	REACTIONS WITH SERA		
	ANTI-rh'	ANTI-rh''	ANTI-Rh ₀		ANTI-rh'	ANTI-rh''	ANTI-Rh ₀
Rh ₀	—	—	+	rh	—	—	—
Rh ₁	+	—	+	rh'	+	—	—
Rh ₂	—	+	+	rh''	—	+	—
Rh ₃ (Rh ₁ Rh ₂)	+	+	+	rh _y (rh'rh'')	+	+	—

*Type Rh₁ contains the two factors Rh₀ and rh', the name Rh₁ being short for Rh₀rh'; similarly Rh₂ is short for Rh₀rh'' and Rh₃Rh₂ is short Rh₀Rh₀rh''.

Hr factors: Factors of Rh-Hr agglutinogens, which are responsible for reactions with Hr antisera. They are reciprocally related to the Rh factors, for which reason Levine first used the symbol "Hr" to indicate this relationship.

Hr sensitization: The process of becoming sensitized to one or more of the Hr factors. When the Hr factor is not specified, it is usually the hr' factor which is intended, since this is by far the most antigenic, and the most important clinically.

Hr testing: Examination of the blood with Hr antiserum. When the exact serum is not indicated, it is usually anti-hr' serum which is intended.

Rh typing: Classification of blood within one of the eight Rh types with the aid of all three Rh antisera (see Table 111): anti-Rh₀, anti-rh', and anti-rh''. There are four Rh₀-positive types and four Rh₀-negative types. For blood transfusions to all Rh-negative recipients, only type rh (Rh negative by the use of all three Rh antisera) should be given, to avoid isoimmunization due to injection of blood containing rh' or rh'' factors. All recipients who belong to types rh' or rh'' must be given Rh-negative blood (type rh); since they lack the most antigenic of the Rh factors, Rh₀, they must not receive Rh₀-positive blood in a transfusion. All donors who are Rh₀-negative but rh'- or rh''-positive must give their blood only to Rh-positive individuals.

Univalent antibodies: One form in which antibodies of any given specificity may occur. Such antibodies tend to appear usually late in the course of immunization, are relatively thermostable, and readily pass through the intact placenta. In saline media, univalent Rh₀ antibodies combine with and coat Rh-positive cells without clumping them. In protein media or in the presence of high-molecular colloids, univalent Rh antibodies clump cells containing the corresponding specific blood factor.

Bivalent antibodies: Another form in which antibodies of a given specificity may occur. Such antibodies usually are produced early in the course of immunization, are relatively thermolabile, and are effectively held back by the intact placental barrier. In saline as well as colloidal media, they are capable of directly clumping cells possessing the corresponding specific antigen.

The differences between the bivalent and the univalent antibodies are summarized in Table 106.

Agglutination: Specific clumping of cells produced by bivalent antibodies.

Conglutination: Specific clumping of cells produced by the combined action of univalent antibodies and congrutinin.

Conglutinin: A colloidal aggregate of serum proteins which is adsorbed by cells that have been sensitized by their specific univalent antibody, but not by cells that have not been sensitized, and after adsorption, causes the cells to stick together. Conglutinin is relatively thermostable, but is sensitive to dilution with crystalline solutions, which causes it to dissociate into its constituent molecules. It is related to complement.

Conglutinin substitutes: Surface colloids, such as acacia, gelatin, dextran, and PVP, of complex molecular structure which can take the place of plasma in the conglutination test. Their ability to produce conglutination is due to their cohesive and adhesive properties, but they have the disadvantage of causing cells not coated by glutinin to clump weakly, an undesirable quality not exhibited by natural or fortified conglutinin, and therefore it is dangerous to use these substitutes.

Fortified conglutinin: Conglutinin which has been improved by the addition of the optimal amounts of albumin. Appropriate amounts (one-fourth to one-third its volume) of 30% albumin mixed with plasma contribute to the colloidal aggregates of protein in the plasma and thus enhance the conglutinin effect.

Rh antisera, anti-Rh sera, anti-rhesus sera: Sera containing Rh or Hr antibodies, or both. These antibodies may occur singly, such as anti-Rh₀, anti-rh', anti-rh'', anti-rh^w, anti-hr, anti-hr', and anti-hr'', or in combinations, such as anti-Rh₀', anti-Rh₀'', anti-Rh₀''' anti-rh'', hr', and anti-rh', hr''.

Rh agglutinating serum: Serum containing Rh antibodies predominately of the bivalent variety. Such antisera usually give best results by the so-called tube agglutination method in which dilute saline suspensions of red cells are mixed with the testing serum in small tubes, and the reactions read after incubation at body temperature for 60 to 120 minutes.

Rh conglutinating serum: Serum containing Rh antibodies predominately of the univalent variety. Such sera give best results by a variety of technics utilizing the principles of conglutination. For example, in the slide method a drop of serum is mixed with a drop of the whole, undiluted, oxalated blood being tested, and the reaction read with the naked eye after the slide has been tilted back and forth for a minute or two over a warmed viewing box. Tube technics may also be used, for example, by using diluted suspensions of cells in plasma or albumin instead of saline. It is possible that all Rh antisera contain a mixture of both univalent and bivalent antibodies. If the univalent antibodies predominate, the antiserum must be used by a conglutination method; if bivalent antibodies predominate, the tube agglutination test may be used. If antiserum containing a suitably balanced mixture of Rh₀ agglutinating and blocking antibodies is titrated in saline medium, a prozone may occur.

Rh sensitization; Rh immunization: The act of becoming sensitized to the Rh factor. This may occur in an Rh-negative individual as a result of a transfusion or an injection of Rh-positive blood or as the result of a pregnancy with an Rh-positive fetus. Injections of small doses (2 c.c.) of Rh-positive blood into Rh-negative individuals, at four-month intervals, cause more than 40% of subjects to become immunized (for preparation of anti-Rh serum). When injections are continued, more than 75% of Rh-negative persons can be immunized. Clinically, two widely spaced transfusions of Rh-positive blood into Rh-negative persons are more likely to lead to Rh sensitization than multiple transfusions in a short space of time. Fetal blood enters the maternal circulation during the course of approximately one out of three normal pregnancies, and one out of three deliveries. Once a person has been sensitized to the Rh factor, he remains so for the remainder of his life, although the titer gradually becomes weaker. When this occurs, a small injection of Rh-positive blood will stimulate a pronounced and sustained rise in antibody titer.

Rh agglutination test: A test to detect Rh antibodies of the bivalent type. When applied to sera from Rh-negative individuals being tested for Rh sensitization, a drop of the patient's whole serum is mixed with a drop of 2% saline suspension of the three types of Rh-positive red cells (group O) and the reactions are read after the mixture has been incubated at body temperature for 1 hour. See Fig. 245.

Rh blocking test: A test to detect Rh antibodies of the univalent variety. This test is also known as the indirect test for univalent Rh antibodies. The patient's whole serum or diluted serum is first mixed with a 2% saline suspension of Rh-positive red cells as in the agglutination test. If no clumping occurs a drop of good anti-Rh₀ agglutinating serum of moderate titer is added and the mixture incubated

1 or 2 hours at body temperature. See Fig. 245. The failure of clumping to occur despite the addition of anti-Rh₀ agglutinating serum, or a distinct weakening of the reaction, indicates the presence of Rh₀ blocking antibodies.

COMPARISON OF Rh AGGLUTINATION AND BLOCKING REACTION

(Test in Saline Media)

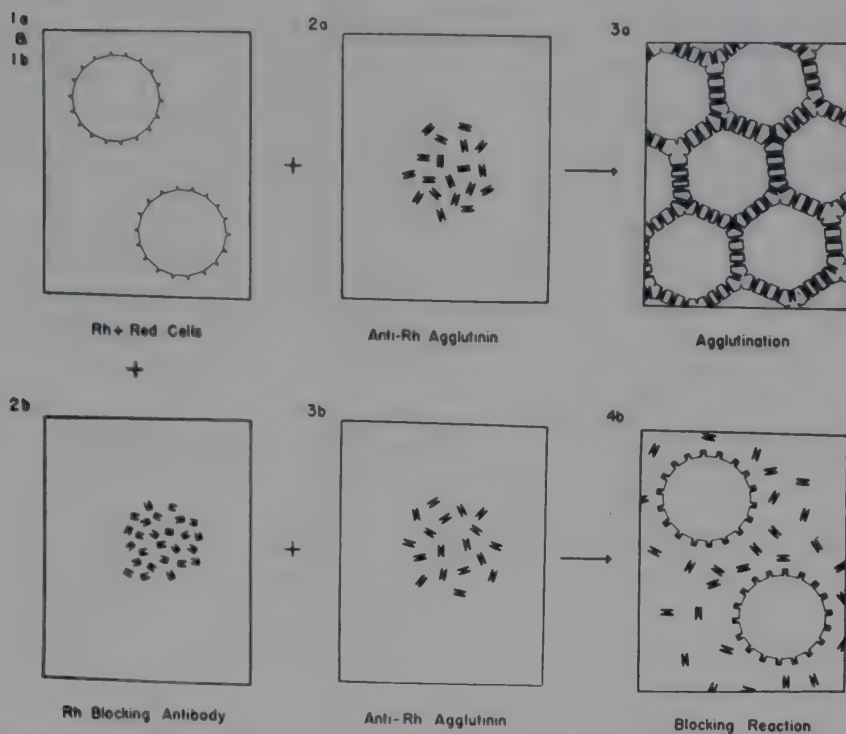


Fig. 245.—(Courtesy A. S. Wiener: Lab. Digest 14: No. 6, 1950.)

Rh CONGLUTINATION REACTION

(Tests in Plasma or Serum Media)

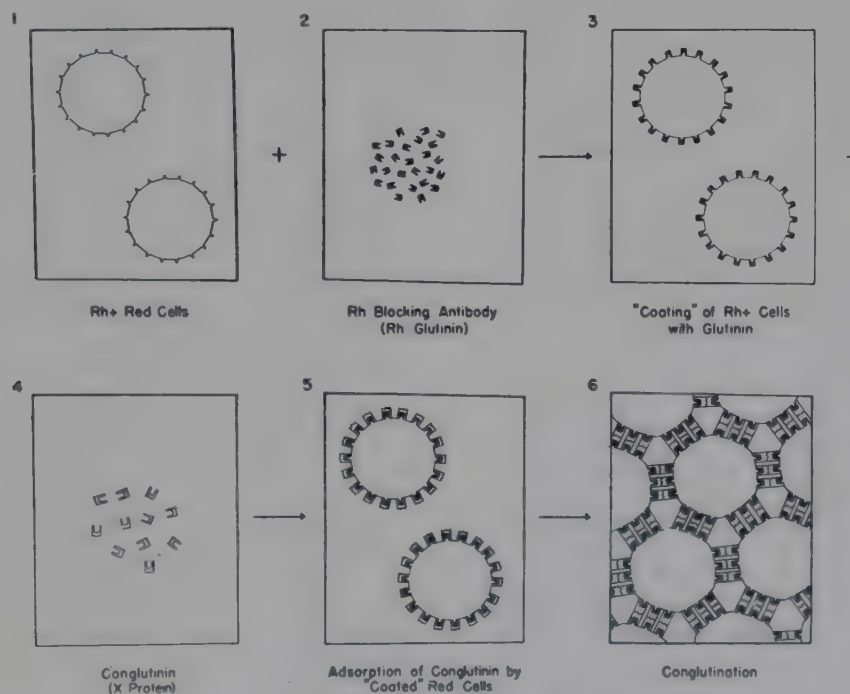


Fig. 246.—(Courtesy A. S. Wiener: Lab. Digest 14: No. 6, 1950.)

Rh conglutination test: Another test for Rh antibodies of the univalent variety, also known as the direct test for univalent Rh antibodies. When applied to the patient's serum, the initial stage of the test is the same as for the agglutination or blocking tests. After the red cells have sedimented, however, the supernatant fluid is removed as completely as possible and replaced by a drop of albumin-plasma mixture

(3 or 4 parts pooled plasma and 1 part 30% bovine albumin). The cells are re-suspended and the reactions read after another period of incubation. The technic can be carried out in a tube in a single step, by using test cells suspended in 20% albumin solution. The conglutination test, when properly performed, is 5 to 40 times as sensitive as the blocking test for detecting univalent antibodies. Other modifications of the test have also been devised. See Fig. 246.

Antiglobulin test, "developing" test, indirect Coombs test for Rh antibodies: Another method for testing for univalent Rh antibodies. The test depends on the fact that the univalent antibodies coating the red cells are human serum globulin. A precipitating rabbit antiserum, prepared by injecting human serum or purified human serum globulin into rabbits (Coombs serum, commercially available), is first absorbed with pooled washed human red cells to remove heteroagglutinins, and is then diluted to optimum strength. The patient's whole or diluted serum is allowed to react with a saline suspension of Rh-positive cells (group O). The sensitized cells are then washed three times with a large volume of saline to remove the supernatant human serum proteins as completely as possible. A drop of Coombs serum is then added, and the mixture centrifuged at 500 r.p.m. for 2 minutes. If the cells are then clumped, this indicates that they have been sensitized by univalent antibodies; failure to clump indicates the absence of sensitization. See Fig. 247.

ANTIGLOBULIN METHOD OF TESTING FOR UNIVALENT Rh ANTIBODIES

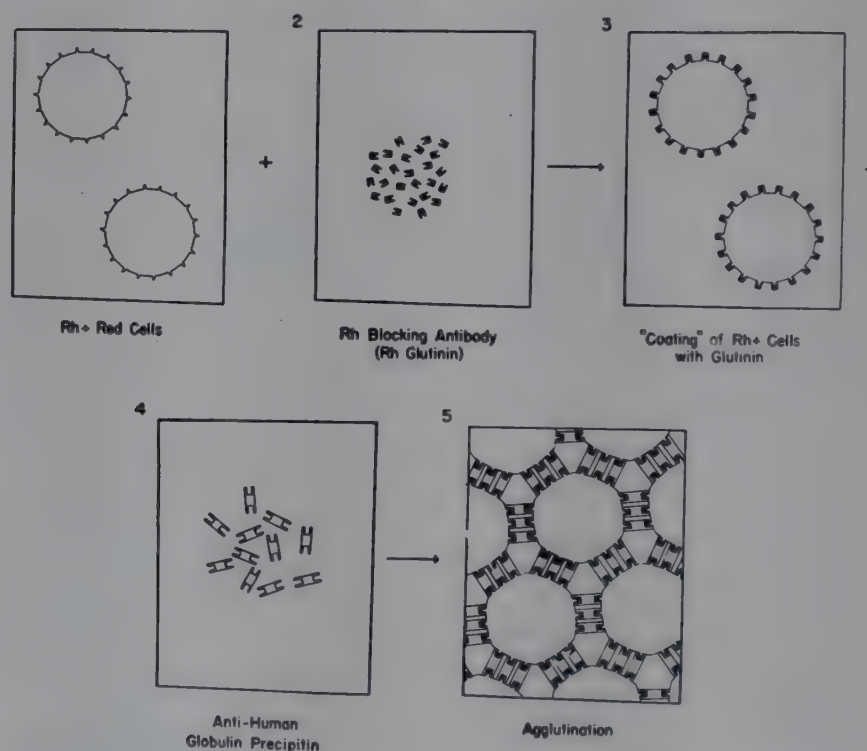


Fig. 247.—(Courtesy A. S. Wiener: Lab. Digest 14: No. 6, 1950.)

Rh antibody tests with enzyme-treated cells: If group O, Rh-positive red cells are washed three times to remove enzyme inhibitor and then treated with proteolytic enzymes like trypsin or papain, they become agglutinable by antisera containing the corresponding univalent Rh-Hr antibody. This test detects the same antibody as the blocking test, the conglutination test, and the antiglobulin test. The test with enzyme-treated cells equals the other tests in specificity and exceeds them in sensitivity. If the sensitivity of the blocking test can be rated as 1, then the sensitivity of the albumin-plasma conglutination test could be rated as approximately 30, the sensitivity of the anti-globulin test as approximately 50, while the sensitivity of tests done with enzyme-treated cells could be rated as approximately 100. The reactions obtainable in the tests with enzyme-treated cells are indicated diagrammatically in Fig. 248.

Titration: Determining the antibody in an antiserum. Unlike chemical titrations, serologic titrations have a large intrinsic error, so that variations up to 100% or 200% are not unusual even in the hands of experts. In the usual technics, the test cells are mixed with progressively doubled dilutions of the serum being examined in a series of tubes, and the highest dilution giving a distinct positive reaction determined.

Titer of a serum: The amount of specific antibody in an antiserum; the strength of a serum. This is usually expressed in units, which are best calculated by taking the reciprocal of the highest dilution giving a so-called one-plus reaction. When determining the titer of a testing serum, the method of titration used should correspond to the method used in the actual tests, if the titer value is to have any significance. The literature contains many reports of fantastically high titers, running into the millions, due in part to "carrying over" and in part to selecting vague reactions as the end point. To avoid such blunders, and in view of the intrinsic inaccuracy of the method, one should calculate the titer values by taking the average of many tests done on different days with many different test cells.

CLUMPING OF ENZYME-TREATED Rh-POSITIVE CELLS BY UNIVALENT Rh ANTIBODIES

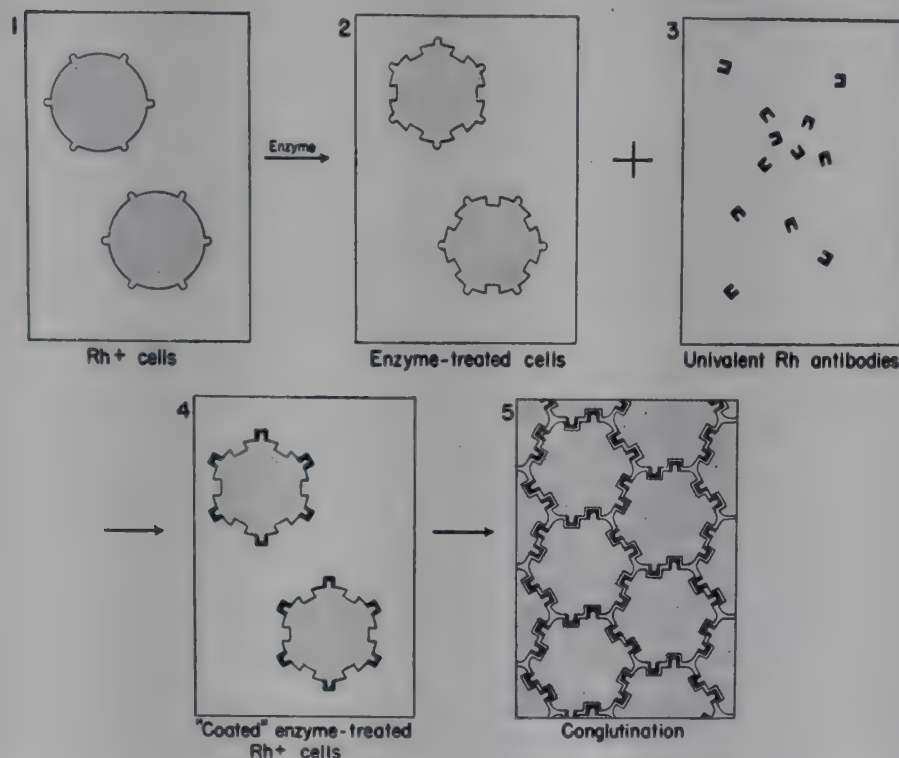


Fig. 248.—(Courtesy A. S. Wiener: Lab. Digest 14: No. 6, 1950.)

Rh₀ blocking serum: An Rh₀ antiserum containing univalent Rh antibodies of sufficiently high titer to give a distinct blocking reaction. Depending on the method used, an Rh₀ antiserum must contain a minimum of 5 to 40 units of univalent antibody, as determined by the conglutination method, in order to give a distinct blocking reaction.

Phenotypes: The characteristics of individuals of a species as determined by direct observation, measurement, chemical or serologic testing, etc. Thus, the characteristics, blue eyes, tall, short, harelip, hemophilic, group O, knock-knees, Rh₁Rh₂, etc., all represent phenotypic characteristics. The phenotype may be partly or wholly genetically determined, or may be entirely determined by the environmental influences; e.g., the color of the hair is usually genetically determined, but in some individuals it may merely be the result of beauty treatment.

Rh typing: Classification of individuals within one of the eight Rh types with the aid of anti-Rh₀, anti-rh', and anti-rh'' sera. Note the distinction between "Rh-typing" and "Rh testing."

Genotypes: The constitutional make-up of an individual, as determined by heredity. The genotype is transmitted through the germ cells by means of their chromosomes, although there is evidence also, in rare exceptional cases, of cytoplasmic inheritance. According to the gene theory, each of the 24 pairs of chromosomes contains numerous genes arranged in linear order. Every individual has a pair of each kind of genes, one derived from the maternal parent and the other from the paternal parent. The make-up of the individual in terms of these genes is known as his "genotype."

Rh₀ phenotypes: The two phenotypes, Rh positive and Rh negative, can be distinguished by using anti-Rh₀ serum.

Rh₀ genotypes: The three genotypes *RhRh*, *Rhrh*, and *rhrh* are based on Landsteiner and Wiener's theory that the **Rh₀** factor is inherited as a simple Mendelian dominant by means of a pair of allelic genes, *Rh* and *rh*. Thus, Rh-negative individuals are always homozygous (*rhrh*). When two Rh-negative individuals marry, they produce only Rh-negative children. Rh-positive individuals may be either homozygous (*RhRh*) or heterozygous (*Rhrh*).

Homozygous: Genetically pure, with genotype consisting of two identical genes. If an Rh-negative expectant mother has a homozygous Rh-positive husband, all the children will be Rh positive.

Heterozygous: Genetically impure, with genotype consisting of two different genes. If an Rh-negative expectant mother has a heterozygous Rh-positive husband, there is 50% chance that the children will be Rh positive and 50% chance that they will be Rh negative, or, as usually stated, 50% of the children will be Rh positive and 50% will be Rh negative.

Rh genes: The series of allelic genes which determine the various sorts of Rh agglutinogens and Rh blood types (see Table 107). Eight distinct varieties have been completely identified, namely, *r*, *r'*, *r''*, *r^s*, *R⁰*, *R¹*, *R²*, and *R^z*. These genes each determine corresponding agglutinogens designated as *rh*, *rh'*, *rh''*, *rh_s*, *Rh₀*, *Rh₁*, *Rh₂*, and *Rh_z*. Note that in order to avoid ambiguity between symbols for genes and agglutinogens, italics are used and the letter "h" is dropped when designating genes. Also, while superscripts and subscripts are both used in the symbols for agglutinogens, only superscripts are used in the symbols for the genes in order to conform with the accepted practice of geneticists. Also note that, in addition to the shorthand symbols *Rh₁* and *Rh₂* (short for *Rh'₀* and *Rh''₀*, respectively), the symbol *rh_s* is used as an abbreviation for *rh'''*, while *Rh_z* is short for *Rh'₀''*. The most common of the eight Rh genes among Caucasoids are *r*, *R¹*, and *R²*, while *R⁰*, *r'*, and *r''* are relatively rare, and *r^s* and *R^z* are extremely rare, so that the last two can usually be disregarded. On the other hand, *R⁰* is the most common gene in Negroids, while *R^z* has its maximum frequency of about 3 to 5% among Mongoloids. When discussing only the results of tests with Rh₀ serum, which divides people into two types, Rh positive and Rh negative, only one pair of genes need be considered, namely, *Rh* and *rh*. Obviously, gene *Rh* comprises four genes, *R⁰*, *R¹*, *R²*, and *R^z*, while gene *rh* comprises four genes, *r*, *r'*, *r''*, and *r^s*.

Allelic genes; allelomorphic genes: Alternative genes situated at corresponding loci in a pair of chromosomes. Thus genes which influence specific characters such as the blood group, eye color, etc., are located at a specific locus on a pair of chromosomes. All the alternate genes which can be located at a given locus are known as allelic. Since the locus for the allelic genes of the A-B-O groups is on a different pair of chromosomes from the allelic genes of the M-N-S types, while the allelic genes of the Rh-Hr system are on a third pair of chromosomes, these three blood group systems are inherited independently of each other. (The Rh locus is not on the sex chromosomes.) When nonallelic genes for two distinct characteristics are on the same pairs of chromosomes, they are said to be **linked**; if they are on different chromosomes, they are said to be **independent**.

Subtyping for rh^w factor: By using anti-rh^w serum, a subdivision of the eight Rh types can be made. Factor rh^w occurs in approximately 5% of Caucasoids, and always in association with factor rh'. Thus type Rh₁ (Rh'₀) is subdivided into Rh₁^w and Rh₁, proper, type Rh₁Rh₂ into Rh₁^wRh₂ and Rh₁Rh₂, proper, and one or two examples of

rare type rh'^w have been found. A similar subdivision of type $rh'rh''$ is theoretically possible. The practical importance of the rh^w factor is to explain rare puzzling examples of erythroblastosis fetalis when both mother and baby are type Rh_1 , where the baby is subtype Rh_1^w and the mother has been sensitized to the rh^w factor.

Anti-Hr sera: Antisera specific for the Hr factors. These are much rarer than anti-Rh sera, since the Hr factors are usually less antigenic than the Rh factors. Three anti-Hr sera are available: anti- hr' , anti- hr'' , and anti- hr . The principal practical value of anti- hr' serum is to subdivide type Rh_1 into the two types Rh_1Rh_1 and Rh_1rh , and the main value of anti- hr'' serum is to subdivide type Rh_2 into Rh_2Rh_2 and Rh_2rh . This gives a presumptive diagnosis of homozygosity or heterozygosity, and aids in anticipating the possible occurrence of erythroblastosis in the unborn child. Anti- hr'' sera are much rarer than anti- hr' . Anti- Hr_0 serum has not yet been found, though a different serum anti- hr has been found.

Rh-Hr types, Rh-Hr phenotypes: Types of human blood demonstrable by the combined use of Rh and Hr antisera. The number of phenotypes which can be distinguished depends on the number of antisera used in the tests, and the evolution of our knowledge as more of the antisera became available is summarized in Table 111. When all three Hr sera as well as the three Rh sera are used, 27 Rh-Hr types can be distinguished corresponding to the 36 theoretically possible genotypes. Since Hr_0 serum has still not been discovered, the examination will usually be confined to tests for hr' , and occasionally for hr'' , in which only 18 phenotypes can be distinguished. It should be emphasized again that many of the phenotypes are quite rare, especially those involving the agglutinogens rh_1 and Rh_2 . In order that the symbols may indicate what tests have actually been made, the "h" is retained when tests have been made for hr' and hr'' only, while the "h" is dropped when tests with Hr_0 have also been made. In selecting the notations for the Rh-Hr type, the principle is employed that the first symbol shall represent the reactions with the Rh antisera, while the second symbol represents the reactions with the reciprocally related Hr antisera. For example, the phenotype Rh_2rh would represent blood reacting with Rh antisera, anti- Rh_0 and anti- rh'' but not with anti- rh' ($Rh_2 = Rh_0 + rh''$), and with Hr antisera anti- hr' and anti- hr'' .

The phenotype corresponding to any given genotype is determined by adding together the effects of the two genes which make up the genotype. For example, if it is desired to know the reactions given by the blood of an individual of genotype R^2r' , one proceeds as follows:

		REACTIONS WITH Rh SERUM			REACTION WITH Hr SERUM		
		Anti- Rh_0	Anti- rh'	Anti- rh''	Anti- Hr_0 (hypothetical)	Anti- hr'	Anti- hr''
Gene R^2	=	+	-	+	-	+	-
Gene r'	=	-	+	-	+	-	+
Therefore by addition genotype R^2r'	=	+	+	+	+	+	+

The phenotype name for blood giving positive reactions with all three Rh sera and all three Hr sera is R_2rh , but since anti- Hr_0 serum to date exists only hypothetically, the name Rh_2Rh_0 is used. If tests are made only with the three Rh antisera, the name Rh_2 must be used.

Rh variants: Some blood specimens give weak or intermediate reactions with one or more of the Rh-Hr antisera, demonstrating the existence of variants of the Rh-Hr factors. Of these, the most important are the variants of the Rh_0 factor, designated \mathfrak{Rh}_0 . \mathfrak{Rh}_0 factor may occur in the absence of factors rh' and rh'' (type \mathfrak{Rh}_0), or in association with rh' but not rh'' (type \mathfrak{Rh}_1), or in association with factor rh'' but not rh' (type \mathfrak{Rh}_2). Rh_0 variants are rare, and occur far more frequently among Negroids than among Caucasoids. In Caucasoids, as many as half of the specimens formerly typed as rh' are now known to belong to type \mathfrak{Rh}_1 . The three agglutinogens \mathfrak{Rh}_0 , \mathfrak{Rh}_1 , and \mathfrak{Rh}_2 are inherited by corresponding allelic genes \mathfrak{R}^0 , \mathfrak{R}^1 and \mathfrak{R}^2 .

TABLE 106.—PROPERTIES OF UNIVALENT AND BIVALENT ANTIBODIES¹

CHARACTERISTIC	BIVALENT ANTIBODIES	UNIVALENT ANTIBODIES
Common name	Agglutinin, precipitin, agglutinating antibody, complete antibody	Glutinin, blocking antibody, conglutinating antibody, incomplete antibody
Usual time of appearance in course of immunization	Early	Late
Resistance to heating	Relatively thermolabile	Relatively thermostable
Reactions of Rh₀ antibodies* in saline medium	Clumps cells by agglutination	Coats cells without clumping them—blocking reaction
Reactions of Rh₀ antibodies in colloid medium	Clumps cells by agglutination	Clumps cells by conglutination
Reaction in presence of complement	Does not fix complement or lyse cells	Fixes complement and lyses cells if the number of antigenic loci on cells surface is adequate; viz., lysis occurs in A-B-O tests but not in Rh-Hr, Kell, or Duffy tests
Behavior in mixed agglutination tests	Specific clumps are formed	Clumps contain more than one kind of cell
Opsonic effect	None	Positive in presence of complement
Chemical nature	Euglobulin; precipitated by sodium sulphate solutions of concentrations 13.5 to 17.4%	Pseudoglobulin; precipitated by sodium sulphate solutions of concentrations 17.4 to 21.5%
Electrophoretic behavior	Alpha and beta globulins	Gamma globulins
Sedimentation constant	17	7
Probable molecular weight	930,000	155,000
Diffusibility	Poor	Good
Behavior relative to placenta	Held back by intact placenta	Passes through placenta readily
Half-life	Probably 2 weeks or less	30 to 35 days
Role in syphilis	Flocculating antibody	Complement fixing antibody
Role in disease	Precipitating and agglutinating antibody	Protective antibody; antitoxin
Role in allergy	Sensitizing antibody (reagin)	Blocking antibody
Role in erythroblastosis	Not significant	Major

*The in vitro reactions are somewhat different, depending on the antigen-antibody system.

¹From Wiener, A. S.: An Rh-Hr Syllabus, The Types and Their Applications, New York, 1954, Grune & Stratton. By permission of the author and the publisher.

Rh-Hr and C-D-E: Use of the letters C, D, E for the Rh-Hr types is bound to lead to confusion and errors, since these symbols are based on a disproved genetic theory (Wiener)¹ and the misconception that blood factors and agglutinogens are identical. C = rh', D = Rh₀, and E = rh''. Thus type rh is cde, type rh' is Cde, type rh'' is

TABLE 107.—THE Rh SERIES OF ALLELIC GENES

GENES		CORRE- SPONDING AGGLU- TINOGENS	REACTIONS WITH Rh SERUM			REACTIONS WITH Hr SERUM		
DESIGNA- TIONS	FREQUEN- CIES AMONG N.Y.C. WHITE (PER CENT)		ANTI-Rh ₀	ANTI-rh'	ANTI-rh''	ANTI-hr'	ANTI-hr''	ANTI-hr
r	38.0	rh	—	—	—	+	+	+
r'	1.4	rh'	—	+	—	—	+	—
r''	0.5	rh''	—	—	+	+	—	—
r ^v	0.01	rh _v	—	+	+	—	—	—
R ⁰	3.2	Rh ₀	+	—	—	+	+	+
R ¹	40.4	Rh ₁	+	+	—	—	+	—
R ²	16.4	Rh ₂	+	—	+	+	—	—
R ^z	0.1	Rh _z	+	+	+	—	—	—

TABLE 108.—PRESENT STATUS OF THE Rh-Hr BLOOD TYPES

GENE	CORRE- SPONDING AGGLU- TINOGEN	REACTIONS WITH Rh SERA				REACTIONS WITH Hr SERA		
		ANTI- Rh ₀	ANTI- rh'	ANTI- rh''	ANTI- rh ^w	ANTI- hr'	ANTI- hr''	ANTI- hr
r	rh	—	—	—	—	+	+	+
r'	rh'	—	+	—	—	—	+	—
r' ^w	rh' ^w	—	+	—	—	—	+	—
r''	rh''	—	—	+	—	+	—	—
r ^v	rh _v	—	+	+	—	—	—	—
R ⁰	Rh ₀	+	—	—	—	+	+	+
R ¹	Rh ₁	+	+	—	—	—	+	—
R ²	Rh ₂	+	—	+	—	+	—	—
R ^z	Rh _z	+	+	+	—	—	—	—
R ^{1w}	Rh ₁ ^v	+	+	—	+	—	+	—
R ⁰	Rh ₀	weak	—	—	—	+	+	+
R ¹	Rh ₁	weak	+	—	—	—	+	—
R ²	Rh ₂	weak	—	+	—	+	—	—

TABLE 109.—EIGHT Rh TYPES AND THEIR TWENTY-ONE GENOTYPES*

Rh BLOOD TYPES	APPROXIMATE FREQUENCY IN N.Y.C. (%)	POSSIBLE GENOTYPES
rh	13.0	rr
rh'	1.5	r'r' and r'r
rh''	0.5	r''r'' and r''r
rh'rh''	0.01	r'r''
Rh ₀	2.5	R ⁰ R ⁰ and R ⁰ r
Rh ₁	52.5	R ¹ R ¹ , R ¹ r', R ¹ r, R ¹ R ⁰ , and r'R ⁰
Rh ₂	15.5	R ² R ² , R ² r'', R ² r, R ² R ⁰ , and r''R ⁰
Rh ₁ Rh ₂	14.5	R ¹ R ² , R ¹ r'', and r'R ²

*Omitting genes r^v and R^z.

cdE, type rh_v is CdE, type Rh₀ is cDe, type Rh₁ is CDe, type Rh₂ is cDE, and type Rh_z is CDE. Much confusion exists in the designation of the genotypes with this nomenclature, and it is suggested that it be dropped from the literature.

The Rh antigen was believed at first to be inherited on the basis of a single gene *Rh* and its allele *rh*. As already indicated, Wiener¹ postulated

¹Wiener, A. S.: Science, p. 595, 1944.

that there were actually six allelic genes which might possibly occupy a single locus in the chromosome. In 1941 Levine² described on the basis of the antibody in the serum of the blood what was essentially just the opposite to Rh and he called it Hr. The necessity of including this antigen within the sphere of the Rh antigen led to the possibility of another theory by Fisher and Race.³ However, this theory had previously been disproved and discarded by Wiener.

In an editorial^{3a} J. M. Hill discussed this entire question. He advised that in the light of present knowledge it seems best to abandon all concepts of dominance of Rh genes whether relative or absolute and simply consider all Rh types as determined by allelomorphic genes without dominance.

TABLE 110.—THE Rh-Hr BLOOD TYPES AND THEIR CORRESPONDING GENOTYPES

Rh BLOOD TYPES†	REACTION WITH SERUM			Rh SUBTYPES†	APPROX- IMATE FREQUENCY IN N.Y.C. (%)	POSSIBLE GENOTYPES
	ANTI- hr'	ANTI- hr''	ANTI- hr			
rh	*	*	*	rh	13.0	<i>rr</i>
rh' {	—	*	—	rh'rh'	0.01	<i>r'r'</i>
rh'' {	+	*	+	rh'rh	1.0	<i>r'r</i>
rh'rh'' {	*	—	—	rh''rh''	0.005	<i>r''r''</i>
Rh ₀	*	+	+	rh''rh	0.5	<i>r''r</i>
Rh ₁ {	*	*	—	rh'rh''	0.01	<i>r'r''</i>
Rh ₂ {	—	*	—	Rh ₀	2.0	<i>R⁰E⁰ and R⁰r</i>
Rh ₁ Rh ₂ {	+	*	+	Rh ₁ Rh ₁	20.0	<i>R¹R¹ and R¹r'</i>
	*	—	—	Rh ₁ rh	34.0	<i>R¹r, R¹E⁰, and R⁰r'</i>
	*	+	+	Rh ₂ Rh ₂	3.0	<i>R²R² and R²r''</i>
	*	*	—	Rh ₂ rh	12.0	<i>R²r, R²E², and R⁰r''</i>
	*	*	—	Rh ₁ Rh ₂	14.5	<i>R¹R², R¹r'', and r'R²</i>

*Tests which need not be made because the reactions in these combinations are invariably positive, except for types rh'rh'' and Rh₁Rh₂ in the case of individuals carrying the rare genes *r''* and *R²*. Should these tests be done and negative reactions obtained, there has been an error in technic, and the entire examination should be repeated.

†These are phenotypes and the symbols have been selected so as to indicate what tests have actually been done and what reactions were obtained. For example, type Rh₁ indicates that tests have been made only with sera anti-rh', anti-rh'', and anti-Rh₀, and positive reactions obtained with anti-rh' and anti-Rh₀ and a negative reaction with anti-rh''; on the other hand, type Rh₁Rh₁ indicates type Rh₁ blood which also has been tested with anti-hr' serum with which it gives a negative reaction.

Professor R. A. Fisher's synthesis of the results of the British work on the isolation of Rh genes was briefly described by Race (1944). The Fisher-Race theory proposes that there are three possibly unlike genes which determine the Hr and Rh phenotypes of an individual. These genes are designated *C-c*, *D-d*, *E-e*, and the appropriate combinations of these result in the definite type of individual.

Coombs, Mourant, and Race⁴ have discussed Fisher's views very completely in an article entitled, "A New Test for the Detection of Weak and Incomplete Rh Agglutinins." They stated that Fisher, noticing the mutually antithetical reactions of two of the four distinct types of sera then known (Race, Taylor, Cappell, and McFarlane, 1944) supposed that these two sera were reacting with allelic antigens. One of the antigen Fisher called *C*, and its allele *c*. The two remaining sera did not give antithetical reactions, so the antigens which they recognized were not allelic to each other though they presumably each had an

²Levine, P.: Yearbook of Pathology, pp. 508-513, 1941.

³Fisher, R. A., and Race, R. R.: Nature 157: 48, 1946.

^{3a}Hill, J. M. Am. J. Clin. Path. 17: 494-501, 1947.

⁴Coombs, R. R. A., Mourant, A. E., and Race, R. R.: Brit. J. Exper. Path. 26: 255-266, 1945.

INTERNATIONAL NOMENCLATURE

INTERNATIONAL NOMENCLATURE

2 Rh phenotypes			12 Rh phenotypes				28 Rh-Hr phenotypes				55 Genotypes*																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																									
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* This table does not include hypothetical genes R^{2w} and R^{2y} , which, if they exist at all, are very rare.
† In this table Rh₁ is used as a short designation for Rh₀; Rh₂ is short for Rh₀; rh_y is short for rh''; and Rh₂ is short for Rh₀.

† The reduction in the frequency of type rh' as compared with that given in earlier charts can be attributed to recognition of bloods of type Rh₁ (containing Rh₀ variant) which are now included in type Rh₁ instead of rh'. The agglutinogens Rh₀, Rh₁, and Rh₂, and their corresponding genes R⁰, R¹, and R², are not

given here, because this would serve unnecessarily to complicate the chart, by increasing the number of possible genotypes to 91. Also, no attempt is made to include certain rare exceptional bloods, such as those lacking both factors rh' and hr', and/or lacking both rh'' and hr'', etc.

§ Based on the estimated gene frequencies, $r = 0.38$, $r' = 0.06$, $r'' = 0.05$, $r^w = 0.001$, $r^y = 0.0006$, $R^0 = 0.27$, $R^1 = 0.41$, $R^2 = 0.15$, $R^2 = 0.02$, and $R^w = 0.2$.

From Wiener, A. S.: Vox Sanguinis, May, 1954.

allele. Instead of seven alleles of the Rh gene at one locus as had previously been proposed, Fisher pictured three adjacent loci each with two alleles, each allele corresponding to an antigen which was capable of reacting with one antibody only, as shown diagrammatically as follows:

Antibodies	Loci on Chromosome	Antibodies
Γ	C or c	γ
Δ	D or d	δ
H	E or e	η

The hypothesis required two more antibodies, δ and η , unknown at that time. Mourant (1945) has found η . Thus Fisher predicted the existence of three antisera reciprocally related to the three principal anti-Rh sera. At the time this prediction was made, one of these antisera, anti-**hr'** (or anti-c) was already known. Of the remaining two, only one, anti-**hr''** (or anti-e) has been shown, with certainty, to exist. Instead of anti-**Hr₀** (anti-d), recently a different antiserum, anti-**hr** (or anti-f) has been found, which was not predicted by Fisher.

TABLE 112.—Rh ANTIBODIES AND AGGLUTINOGENS

Original names Fisher's antigenic or genetic notation Antibodies			Agglutinogenst							
Wiener	Fisher	Cappell	Rh_1 CDe	Rh_2 cDE	rh cde	Rh_0 cDe	rh' Cde	rh'' cdE	Rh_z CDE	rh_y CdE
anti-rh'	Γ	anti-C	+	—	—	—	+	—	+	+
anti- Rh₀	Δ	anti-D	+	+	—	+	—	—	+	—
anti-rh''	H	anti-E	—	+	—	—	—	+	+	+
anti- hr'	γ	anti-c	—	+	+	+	—	+	—	—
anti- Hr₀	δ	anti-d	(—)	(—)	(+)	(—)	(+)	(+)	(—)	(+)
anti-rh''	η	anti-e	+	—	+	+	+	—	—	—
anti- hr	None*	None*	—	—	+	+	—	—	—	—

*No name given by Fisher or Cappell; anti-f used by Rosenfield et al.
†One must not confuse the agglutinogens with the types. The types (phenotypes) being determined by a pair of genes (genotypes) therefore necessarily comprise a pair of agglutinogens.

It may be convenient at this point to summarize the contributions made by British investigators to the field of blood grouping during the past decade. This was initiated in 1942 by Taylor and Race, following publication of the American investigations on the Rh factor. After Taylor's premature death, Race took over the leadership of British work, and soon confirmed and extended American reports on the serology and heredity of the Rh-Hr blood types. By studying difficult problems of multiple sensitization, he and his collaborators discovered the blood factors **rh^w**, *Levay*, and *Lutheran*. These and other contributions of British investigators are well summarized in the book of Race and Sanger, the second edition of which has just appeared.⁵ Mourant, codiscoverer of the *Lewis* factor, has published an important book⁶ summarizing recent advances in anthropologic aspects of the subject. Molli-son, who with Cutbush and Parker discovered the Duffy (**F**) factor, studied exchange transfusion and published a fine book on blood transfusion,⁷ while

⁵Race, R., and Sanger, R.: *Blood Groups in Man*, ed. 2, Oxford, 1954, Blackwell Scientific Publications, Ltd.
⁶Mourant, A. E.: *The Distribution of the Human Blood Groups*, Oxford, 1954, Blackwell Scientific Publications, Ltd.
⁷Mollison, P. L.: *Blood Transfusion in Clinical Medicine*, Oxford, 1951, Blackwell Scientific Publications, Ltd.

Pickles contributed a book on erythroblastosis fetalis.⁸ Finally, Dacie has specialized in the study of autosensitization and has recently published a book on hemolytic anemia.⁹

I. Tests for the Determination of the Rh Factor

(1) Detection of the Rh Factor in Blood Cells

A requisite for accurate and successful Rh testing is an efficient anti-Rh serum. It is unfortunate that while the best source of anti-Rh serum is from women who have given birth to erythroblastotic babies or who have had spontaneous abortions or stillbirths resulting from Rh incompatibility, many of these sera do not contain sufficient anti-Rh agglutinins to be used in routine tests. Potent anti-Rh sera are produced by immunizing normal male professional blood donors (see page 1072).

Disadvantages of sera derived from animals immunized to rhesus monkey blood are (1) if guinea pigs are used, the amount of blood obtainable is small; (2) it is commonly believed that absorption with Rh-negative blood cells is necessary to remove undesirable antibodies of such sera; and (3) these animal sera affect only one of the several kinds of Rh factor, but this happens to be the most common type. To identify all the Rh-positive bloods, tests made with animal sera must be supplemented by tests with other sera.

(A) Slide Method.—Anti-Rh₀, anti-Rh₀', and anti-Rh₀'' agglutinating sera for the slide test for Rh testing of human blood cells are now available commercially.* These sera are produced by immunizing normal male professional blood donors. Anti-Rh₀ serum shows 85 per cent of all bloods tested to be Rh₀-positive. If only anti-Rh₀' serum is used, slightly less than 87 per cent of all bloods tested will be positive. If only the anti-Rh₀'' serum is used, about 85.5 per cent of all bloods will be positive.

When testing recipients, use the anti-Rh₀ serum routinely, and if the individual is Rh negative by this test, give only Rh-negative donor blood in a transfusion. If the blood is Rh positive, give Rh-positive blood.

When testing donors, if all three antisera are used, if the blood is negative using the anti-Rh₀ serum, but positive by either or both of the other two, give this blood only to Rh-positive recipients. If positive by all three tests, of course give this blood only to Rh-positive recipients. If negative by all three antisera (type rh), give to Rh-negative recipients.

Reagents.—

For recipients, use anti-Rh₀ slide (agglutinating) serum; use anti-Rh₀, anti-Rh₀' and anti-Rh₀'' slide serum for donors. (If blood bank blood is used, the exact type of blood of the donor is probably given on the blood bottle. This can be detected by use of the tube-agglutinating method. See below.)

Equipment.—

A special viewing box is used for this test. This box is fitted with an electric light globe which will bring the temperature on the plate to 45° to 47° C. See Fig. 232. Since some time is required to bring the temperature on the glass plate to the proper temperature, the light should be on at all times.

Ordinary glass microscopic slides are used.

*Antisera are available from the Certified Blood Donor Service, Jamaica, N. Y., High Titer Serum Laboratory, New York City, Hyland Laboratories, Los Angeles, Calif., and Yonkers, N. Y., American Hospital Supply Corporation, Chicago, and others.

⁸Pickles, M. M.: *Haemolytic Disease of the Newborn*, Oxford, 1949, Blackwell Scientific Publications, Ltd.

⁹Dacie, J. V.: *The Haemolytic Anemias*, London, 1954, J. & A. Churchill, Ltd.

In addition to the antisera, **whole blood** taken in a special anticoagulant is used. (See page 1005.) If clotted blood is submitted, break up the clot and suspend the cells to a concentration of 50% in their own serum. Do not use blood taken in a crystalline solution (saline, sodium citrate, liquid oxalate, etc.). Blood taken from patients with anemia must be centrifuged and enough plasma removed to bring the cell concentration to 50%.

Technic.—

- Carry out the method for each antiserum used.
- Make a large oval ring, using a red wax pencil, on a glass slide, about 1½ in. by ¾ in. Label with the name of the individual being tested and the serum used. See Fig. 240.
- Place 2 drops of whole blood on the slide.
- Add 1 large drop of antiserum (anti-Rh₀ if this is the recipient).
- Prepare similar slides for each antiserum used to test the blood.
- Mix with a wooden applicator and spread the mixture over the entire surface of the oval ring.
- Place on a preheated viewing box and rock back and forth, observing for clumping or lack of clumping.
- At the end of 3 to 5 minutes, tip the box up and add a drop of saline to the top of the slide so that it runs down into the mixture. Rouleaux will be broken up by the saline, but true clumping will not.
- Read macroscopically only; do not read under a microscope.
- Wait for a minute or two before taking the final reading.
- Report clumping as positive, lack of clumping as negative.

Controls:

At the same time, run duplicate tests using known Rh-positive and Rh-negative whole blood as controls. If these react correctly, the results in the tests may be reported. If the controls give incorrect reactions, do not report the results of the tests until new sera are obtained and the tests have been repeated.

Results Possible:

For the **recipient**, see Table 113; for the **donor**, Table 114.

TABLE 113.—THE RECIPIENT

WHOLE BLOOD PLUS ANTI-Rh ₀ SERUM	CONCLUSIONS
+	Rh ₀ positive: give Rh-positive blood
-	Rh ₀ negative: give Rh-negative blood

TABLE 114.—THE DONOR

WHOLE BLOOD PLUS ANTI-Rh ₀ SERUM	WHOLE BLOOD PLUS ANTI-Rh' ₀ SERUM	WHOLE BLOOD PLUS ANTI-Rh'' ₀ SERUM	CONCLUSIONS
-	-	-	Type rh (Rh negative)
+	+	+	Rh positive containing Rh ₀
-	+	-	Type rh'* { if Rh ₀ is
-	-	+	Type rh''* { absent
-	+	+	Type rh _y * (rh'rh'') {
+	-	-	Error
+	-	+	Error
+	+	-	Error

*Give this blood only to Rh-positive patients. This individual should receive only Rh-negative blood unless the exact type is available. Tests for Rh₀ factor may be made.

(B) Tube Method.—

This test should be made routinely on all Rh-negative patients and on Rh-negative donors.

Reagents.—

Anti-rh' saline agglutinating serum, anti-rh'' saline agglutinating serum, anti-Rh₀ saline agglutinating serum, anti-Rh₀ conglutinating (slide test) serum.

In addition to these sera, a 2 or 3% suspension of the patient's (or donor's) cells in saline is needed. Make the suspension as directed on pages 1005 and 1006, wash once in saline, and resuspend to a 2 or 3% suspension in saline.

Use tubes 7.5 cm. by 8 mm., 4 for each test.

TUBE TEST FOR Rh FACTORS

○ Anti-Rh ₀ slide serum	○ Anti-Rh ₀ slide serum
○ Anti-Rh ₀ tube serum	○ Anti-Rh ₀ tube serum
○ Anti-rh'' tube serum	○ Anti-rh'' tube serum
○ Anti-rh' tube serum	○ Anti-rh' tube serum
Patient's cells	Donor's cells

Water bath 1 hour at 37°C.
If negative with anti-Rh₀ tube serum, do Coombs test in tube with anti-Rh₀ slide serum.

Fig. 249.



Fig. 250.—Tube test for Rh factors in cell suspensions.

Technic.—

Label tubes with the identification of the individual being tested and the name of the serum.

Place 1 drop of the cell suspension to be tested in each of four tubes, in a rack. Use the same dropper held at the same angle so that the drop used in each tube will be approximately the same size.

- Add a drop of anti-rh' saline agglutinating serum to tube 1
- a drop of anti-rh'' saline agglutinating serum to tube 2
- a drop of anti-Rh₀ saline agglutinating serum to tube 3
- a drop of anti-Rh₀ slide agglutinating serum to tube 4

Mix and incubate in a water bath at 37° C. for 1 hour.

Tip the rack and take a preliminary reading from the bottoms of the tubes (see Fig. 251). Negative reactions come down in a pear shape, while positives tend to remain round.

Read over a lighted box, spreading the mixture thin in the tube.

Read under a scanning lens of a microscope, reporting clumping as positive and lack of clumping as negative.

If the result in tube 3 is negative, use *tube 4* for a Coombs test.

Wash the cells in tube 4 three times with saline. After the last washing, remove all traces of the saline. This can be accomplished by centrifuging at high speed, turning the tube upside down after the cells have been packed, and tapping the lip of the tube on a gauze pad while the tube is still upside down.

TABLE 115.—RESULTS POSSIBLE WITH THE TUBE TEST FOR Rh FACTORS

CELLS + ANTI-rh' SERUM	CELLS + ANTI-rh'' SERUM	CELLS + ANTI-Rh ₀ SERUM	CELLS + ANTI-Rh ₀ SLIDE SERUM (PLUS COOMBS TEST)	CONCLUSIONS (Rh TYPE)
-	-	-	$\frac{-}{-}$	rh
+	-	-	$\frac{-}{-}$	rh'
-	+	-	$\frac{-}{-}$	rh''
+	+	-	$\frac{-}{-}$	rh _y
-	-	+		Rh ₀
+	-	+		Rh ₁
-	+	+		Rh ₂
+	+	+		Rh _s
-	-	-	$\frac{+}{-}$	Rh ₀
+	-	-	$\frac{+}{-}$	Rh ₁
-	+	-	$\frac{+}{-}$	Rh ₂
+	+	-	$\frac{+}{-}$	Rh _s

Shake thoroughly, and incubate for 5 minutes in a water bath at 37° C.

Add a drop of Coombs serum.

Mix thoroughly, and centrifuge at 500 to 1,000 r.p.m. for 2 minutes.

Read over a lighted box and under a scanning lens.

If the results in tube 3 (anti-Rh₀ saline agglutinating serum) are positive, do not do the Coombs test on tube 4, because it will always be positive under these circumstances.

If the anti-rh' serum clumps the cells, rh' factor is present.

If the anti-rh'' serum clumps the cells, rh'' factor is present.

If the anti-Rh₀ saline agglutinating serum clumps the cells, Rh₀ factor is present.

If the anti-Rh₀ saline agglutinating serum fails to clump the cells, but the Coombs test is positive in tube 4 (slide agglutinating anti-Rh₀ serum), the Rh₀ variant is present. This is reported thus:

- | This means that the original test was negative and also the Coombs test
- . (in the box).
- + | This means that the original test was negative and the Coombs test was
- positive (in the box).*

Sensitivity tests may be run on the serum to detect isoimmunization to the Rh factor at the same time as the tests for the Rh factor are made. If desired, also make the tests for the Hr factors. The tests for Hr will always be positive on Rh-negative persons; therefore, it is best to wait for the results of the Rh tests before using serum to make tests for the Hr factors.

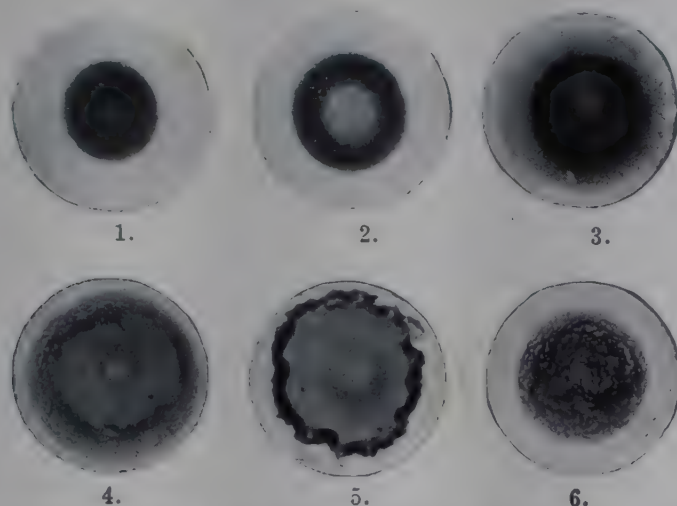


Fig. 251.—Magnification 1:2. 1 and 2, Negative reactions; the inner light disk in 2 is due to slight convexity in the bottom of the tube. 3, Faintly positive reaction. 4, Weak reaction. 5 and 6, Typical positive reactions. (From Landsteiner and Wiener: J. Exper. Med. 74: 312, 1941.)

(2) Test for the Hr Factor in Blood Cells

(A) Open Slide Method.—

Prepare oxalated whole blood as directed on page 1056.

Obtain slide serum (conglutinating serum), anti-hr' and anti-hr''.

Prepare two slides by marking them with a large oval using a red wax pencil. Label properly.

Place 2 drops of oxalated whole blood on a slide.

Add 1 drop of the antiserum, anti-hr' on one slide, anti-hr'' on the other.

Mix each with a separate applicator and spread the area of the oval.

Place on a lighted viewing box, and rock back and forth for 3 to 5 minutes.

Add a drop of saline and let it run down the specimen. If rouleaux are present, they will break up. Read after 2 more minutes.

Report clumping as positive and lack of clumping as negative.

Results Possible: See Table 116.

TABLE 116

ANTI-hr' SERUM PLUS WHOLE BLOOD	ANTI-hr'' SERUM PLUS WHOLE BLOOD	CONCLUSIONS.
-	-	hr' and hr'' negative
+	-	hr' positive
-	+	hr'' positive
+	+	hr'hr'' positive

*After Lester J. Unger, New York City.

(B) Tube Test.—

Only the test using anti-hr' saline agglutinating serum will be given. Prepare one tube for each antiserum.

Place 1 drop of the unknown washed cells suspended to 2 or 3% suspension in saline in the tube.

Add 1 drop of anti-hr' saline agglutinating serum.

Mix and incubate in a water bath at 37° C. for 1 hour.

Take "bottom readings," and then read over a lighted box and under a scanning lens of a microscope.

Report clumping as positive and lack of clumping as negative.

Clumping means that the blood is hr'-positive, and lack of clumping means it is hr'-negative.

It is not necessary to make Hr tests on Rh-negative blood since it will always be hr' and hr'' positive.

Consult Table 111, page 1053, for results if both Rh and Hr tests are made on a specimen of blood.

II. Detection of the Anti-Rh Agglutinin in Blood Serum

There are two distinct types of antibody formed in blood serum as a result of isoimmunization with the Rh agglutinin: (1) an Rh agglutinin; (2) an antibody which when mixed with saline-suspended Rh-positive cells does not agglutinate them. In fact, its presence prevents agglutination of these cells even when they are all exposed to a good anti-Rh serum. This refers to the so-called "blocking" antibody. Rh-positive blood cells, however, will be clumped with the slide and tube tests (pages 1055 and 1056).¹ Wiener found that by the use of normal plasma instead of saline solution as a diluent for the blood cells, such an antibody will clump Rh-positive cells in the test tube. Bovine serum albumin also activates "blocking" antibodies. Serum albumin also has the power to cause the blocking antibody to bring about clumping.² The mechanism by which serum albumin fractions exert their effects is not clear.

**THE BLOCKING ANTIBODY AND THE ZONE PHENOMENON IN HUMAN
ANTI Rh SERUM** (see page 1038)

Wiener and Peters³ showed that sensitization of Rh-negative individuals against the Rh factor can often be detected by in vitro tests for anti-Rh agglutinins in the serum. It was found, however,^{4, 5} that there were many Rh-negative patients, strongly sensitized to the Rh factor, whose plasma did not contain demonstrable anti-Rh agglutinins. These patients had either intragroup hemolytic transfusion reactions or had delivered babies with erythroblastosis fetalis.

In searching for an explanation of the lack of in vitro agglutination by these sera, Wiener mixed the sera with group O Rh-positive cells, and later added known anti-Rh serum to the same test cells. The cells were not agglutinated by the known anti-Rh serum after they had been in contact with the patients' sera. He concluded that the action of the active agglutinin in

¹Diamond, L. K., and Abelson, N. M.: *J. Lab. & Clin. Med.*, **24**: 122, 1945.

²Cameron, J. W., and Diamond, L. K.: *J. Clin. Invest.* **24**: 20, 1945.

³Wiener, A. S., and Peters, H. R.: *Ann. Int. Med.* **13**: 2306, 1940.

⁴Wiener, A. S.: *Arch. Path.* **32**: 227, 1941.

⁵Levine, P., Burnham, L., Katzin, E. M., and Vogel, P.: *Am. J. Obst. & Gynec.* **42**: 925, 1941.

the added serum had been blocked, and designated such antibodies as were present in these patients' sera as "blocking antibodies." Fig. 252 is a diagrammatic representation of the difference between Rh agglutination and blocking reactions.

Tests were carried out on a number of patients with erythroblastotic infants where the usual tests for anti-Rh agglutinins were unsuccessful, and in most of the cases clear-cut blocking reactions were obtained, thus demonstrating the presence of a special type of Rh antibody. The reaction is specific in that it is obtained only with sera from Rh-negative individuals sensitized by the Rh factor, and not with sera from Rh-positive or Rh-negative individuals not sensitized by the Rh factor.

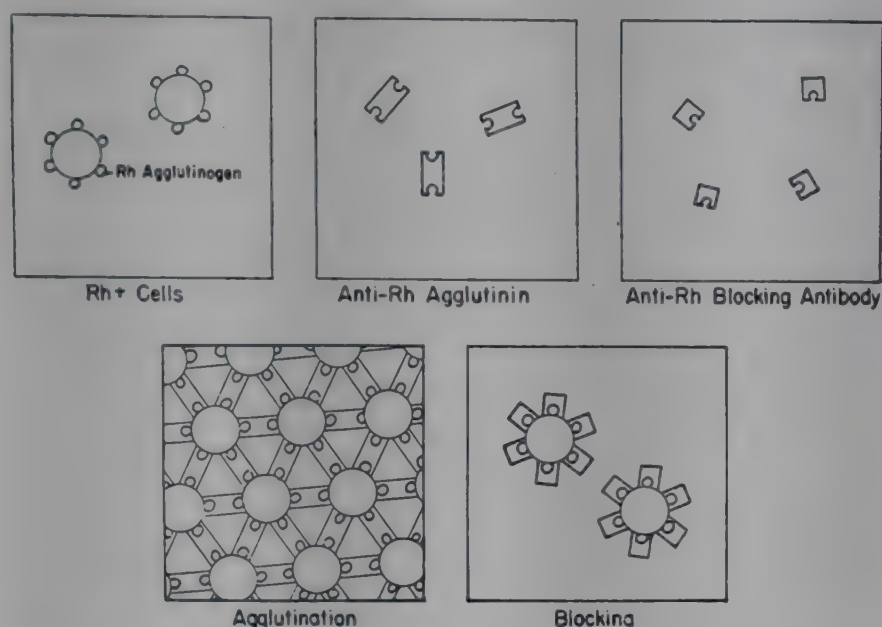


Fig. 252.—Diagrammatic representation of Rh agglutination and blocking reactions. (Wiener, A. S.: *Am. J. Clin. Path.* 15: March, 1945, by permission of the author and publisher, Williams & Wilkins Co.)

A number of human anti-Rh sera have been obtained which exhibit a marked "prozone" effect.^{6, 7} Taylor and associates⁶ attempted to explain the behavior of such sera on the basis of optimal proportions of antigen and antibody. This does not explain why the phenomenon does not occur more frequently. Wiener's experiments suggest that such prozone phenomena are due to the presence in sera of a mixture of blocking and agglutinating antibodies, the agglutinating antibodies being of a higher titer.

Those sera which exhibit the prozone effect cannot be used as diagnostic reagents unless the agglutinin is far more active than the blocking antibody. Experiments seem to indicate that some of these sera can be absorbed with Rh-positive blood, so that after the blocking antibody is specifically removed, the treated serum becomes a useful diagnostic reagent. From a practical standpoint, the significance of these findings lies in the fact that the characteristics of the agglutinating antibody and the blocking antibody are sufficiently clear cut, so that, for example, there is no difficulty in the use of an anti-Rh serum which contains an anti-**rh'** agglutinin and also an anti-**Rh₀** blocking antibody.

⁶Taylor, G. L., Race, A. M., and Ikin, E. W.: *Brit. M. J.* 2: 572, 1942.

⁷Levine, P.: *Arch. Path.* 37: 83, 1944.

It is often necessary to determine the presence in the patient's blood serum of anti-Rh antibodies. Three tests are commonly made: (1) the agglutination test; (2) the blocking test; and (3) the conglutination test. These tests are made simultaneously, and are referred to as "sensitivity" tests.

(1) Sensitivity Test for Rh-Hr Antibodies in Serum Method of Unger

Sensitivity tests for Rh-Hr antibodies should be made of the sera of both the patient and the donors. These tests should be made routinely even when the cross-matching tests are compatible. If present, they might be responsible for post-transfusion reactions if the cells for which they are specific are transfused. This occurs particularly when the patient has **hr'** or other rarer antibodies and receives blood for which the antibodies are specific.

For further discussion, refer to the section on Rh-Hr, pages 1024 ff.

Technic.—(See Fig. 241.)

Have at hand group O Rh_zRh₀ cells. Group O is used because the unknown serum might contain anti-A or anti-B agglutinins which would react with cells derived from groups A, B, or AB, but not with group O. Type Rh_zRh₀ cells are selected because they react with anti-rh', anti-rh'', anti-Rh₀, anti-hr', and anti-hr'' antibodies.

Prepare three cell suspensions:

- (a) Untreated and saline-suspended, 2 or 3% suspension.
- (b) Trypsinated and saline suspended, 2 or 3% suspension.
- (c) Suspended in their own plasma, 5% suspension. Refer to page 1006 for preparation of these suspensions.

Use three tubes, 7.5 cm. by 8 mm. for each serum tested.

Place 1 drop of untreated saline-suspended group O Rh_zRh₀ cells in the first tube, 1 drop of group O Rh_zRh₀ trypsinated cells in the second tube, and 1 drop of group O Rh_zRh₀ cells suspended in their own plasma in tube three.

Add a drop of the serum to be tested to each tube, and shake.

Incubate in a water bath at 37° C. for ½ hour.

Tilt the tubes back and forth gently over a lighted viewing box and observe for clumping or lack of clumping. Read under a scanning lens of a microscope. If any tube appears to have clumps, the individual is sensitized, and has antibodies against the Rh-Hr factors. If all tubes appear to be negative, proceed with the antiglobulin test.

Antiglobulin tests are made on negative-reacting untreated and trypsinated cells, saline-suspended.

Add saline to each tube, to full capacity.

Centrifuge to pack, and pour off the supernatant saline.

Shake and add more saline, centrifuge, remove saline, and shake again. This is the second washing.

Add more saline, and centrifuge to pack the cells firmly. Pour off the supernatant saline and tap the tube upside down against a gauze pad to remove all traces of saline.

Shake to dislodge the cells.

Incubate 5 minutes at 37° C.

Add a drop of antihuman globulin serum (Coombs test) to each tube.

Mix and centrifuge at 500 to 1,000 r.p.m. for 2 minutes.

Gently dislodge the sediment and tip back and forth and read under a scanning lens of a microscope.

If clumping occurs, the individual is sensitized, and an antibody is in the serum specific for a factor present in the clumped cells. Its specificity remains to be determined. If it occurs with saline-suspended cells without the addition of antiglobulin serum, there are agglutinating, bivalent antibodies in the serum. If it occurs with trypsinated cells or upon the addition of anti-

globulin serum, or with cells suspended in their own plasma, but not with saline-suspended cells, there are blocking, or univalent, antibodies present.

If any clumping occurs, this must be reported. If it occurs with donor's serum, reject the donor. If it occurs with patient's serum, report to the director of the laboratory and prepare for an identification and a titration of antibodies.

If no group O Rh₊Rh₀ cells are available, do the 6-tube test, using group O Rh₊, O Rh₊, and O rh untreated and saline suspended cells, and the same series of types but trypsinated and saline suspended. Run Coombs tests on negative tubes containing untreated and trypsinated saline-suspended cells (**Unger test**).

(2) The Conglutination Test for Sensitivity to Rh-Hr Factors

The conglutination test requires that the cells be suspended in their own plasma, and therefore the sensitivity tests of Unger as described above fulfill this requirement. If the Unger method is followed, no further conglutination test need be made.

In the case of Rh-negative pregnant women who have had previous transfusions or pregnancies, Rh antibody titrations should be made before the third month to determine whether antibody is being carried into their present pregnancies. When antibody is demonstrated, titrations should be made at monthly intervals until the seventh month, and at biweekly intervals thereafter until the last month, when they should be done weekly. When the titrations at the third month do not demonstrate Rh antibody, no further studies need be made until the seventh month.

The appearance of antibody or rising titer when antibody is demonstrated at the third month proves that immunity is stimulated and the fetus is Rh positive. An unchanging or falling titer proves that immunity has not been stimulated; the fetus may be Rh positive or Rh negative.

(3) Indirect Test for Blocking Antibodies (Wiener)

Technic.—

Carry out the test in small Kahn test tubes. Use 1 drop of the patient's serum mixed with 1 drop of a saline suspension of group O Rh₊Rh₀ red cells.

Incubate for 1 hour in a water bath at 37° C.

Centrifuge at 1,000 r.p.m. for 1 minute. Read under a scanning lens. If the reaction is positive, anti-Rh agglutinins are present in the patient's serum.

If the reaction is negative, decant all the saline, after centrifugation.

Add to the same tube 1 drop of known anti-Rh₀ agglutinating serum.

Mix and again place in a water bath at 37° C. for 1 hour. Centrifuge and read.

If agglutination now occurs, then the patient's blood serum contained neither anti-Rh₀ agglutinins nor Rh₀ blocking antibodies.

If no agglutination occurs, the patient's serum contains Rh₀ blocking antibodies, otherwise the known Rh-positive cells would have reacted with the added known anti-Rh₀ serum.

(4) Direct Antiglobulin Test of Cord and Infant's Blood* (Also spoken of as the Coombs Test) (for Rh Blocking Antibodies Derived From the Mother)

The antiglobulin test is used routinely whenever antibodies which react to antihuman globulin addition are to be detected. These are the univalent

*This test for coating of the baby's cells by Rh antibodies was first described by Wiener and Sonn: *Am. J. Dis. Child.* 71: 25, 1946. (cf. Wiener, A.S.: *The Rh-Hr Types*, New York, 1954, Grune & Stratton.)

or blocking antibodies. These antibodies are also detected by the blocking, agglutination, and enzyme techniques.

The direct antiglobulin test is also used to detect the presence of univalent (blocking) antibodies coating the red cells of erythroblastotic infants after birth.* These univalent antibodies, when present, have been derived from the isosensitized mother, and are considered the cause of erythroblastosis fetalis. When they are found, the baby is in danger of developing clinical signs of erythroblastosis fetalis, and an exchange transfusion should be performed, using Rh-negative donor blood. (See pages 1075 ff.)

Technic.—

Wash the baby's blood cells three times with saline. After the last washing, tap the tube upside down over a gauze pad to be sure that all the saline has been removed.

Resuspend the cells to approximately 2% suspension in saline.

Place 2 drops of the washed saline-suspended cells in a tube 7 by 70 mm., scrupulously clean. See Fig. 253.

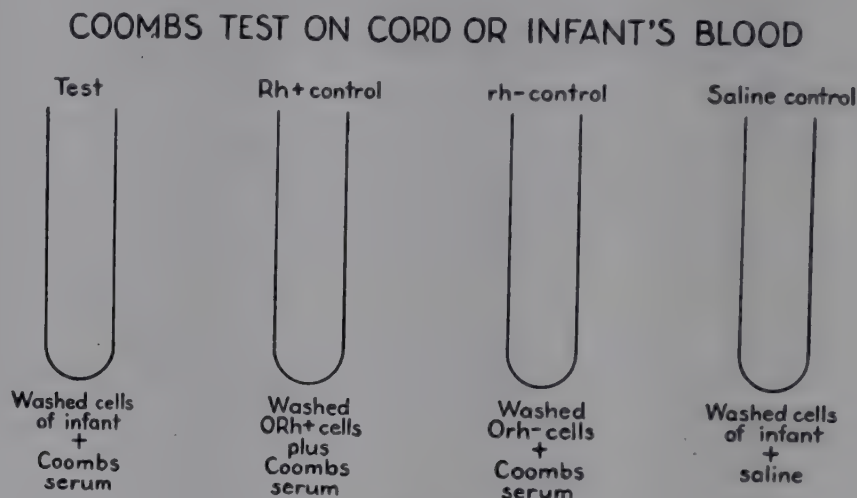


Fig. 253.

Add 2 drops of antihuman globulin (Coombs) serum.

If the cells have been strongly sensitized by the maternal antibodies, clumping will become visible almost immediately. Examine over a lighted viewing box.

If there is only a weak reaction, or if the reaction appears to be negative, allow the tube to stand at room temperature for 15 to 30 minutes.

Centrifuge at 500 r.p.m. for 2 minutes.

Observe for agglutination over a lighted viewing box and under a scanning lens.

Agglutination means that the infant's cells have been coated with blocking antibodies derived from an isosensitized mother. Lack of agglutination means that the baby's cells are not coated.

Controls.—

Controls are made in small tubes.

- (1) 2 drops of washed infant's cells plus 2 drops of saline.
- (2) 2 drops of Rh-positive cells, saline suspended, plus 2 drops of antiglobulin serum.
- (3) If desired, prepare Rh-positive cells by coating them with known blocking antibodies. Add 2 drops of 2% suspension of Rh-positive saline-suspended cells to 2 drops of known Rh blocking serum. Incubate at 37° C. for 30 minutes. There should be no agglutination. Centrifuge and remove the supernatant saline, and wash three times with saline. Add 2 drops of Coombs serum (antiglobulin). The cells should now clump.

*As Wiener has pointed out, coating of the baby's cells can also be demonstrated by the blocking and direct agglutination tests.

(4) 2 drops of Rh-negative cells, plus 2 drops of antiglobulin serum.

Handle the controls in the same manner as the test.

Tubes (1), (2), and (4) should give negative reactions.

The antihuman globulin serum may give a false negative direct or indirect reaction when the red blood cells are coated with a minimal amount of antibody.

Prepare a 1:5 dilution by adding 1 drop of the antihuman globulin to 4 drops of saline. Use 1 drop in the test.

Antihuman globulin serum (Coombs serum) is prepared by hyperimmunization of a rabbit against human serum globulin. Nonspecific antibodies are absorbed from the serum by adding human red cells which have been washed 10 times with saline solution.

(5) Indirect Antiglobulin Test (Coombs Test) for Detection of Isoimmunization¹

See Rh-Hr Sensitivity Testing, page 1062.

(6) Quantitative Direct Antiglobulin Test (Wiener²)

This is a test for measuring the degree of coating of red cells by antibodies from an erythroblastotic infant or a patient with hemolytic anemia.

Wash the red cells to be tested 4 times with an excess of saline, as for the simple direct antiglobulin test.

Titrate the antiglobulin serum against these washed cells.

At the same time titrate the antiglobulin serum against Rh-positive cells which have been maximally coated by sensitizing them with an anti-Rh agglutinating (slide test) serum having a titer well in excess of 16 units by the albumin-plasma method (p. 1080).

Calculate the percentage of coating of the unknown cells by dividing the titer of the antiglobulin serum for these cells by its titer for maximally coated Rh-positive cells, and then multiplying by 100.

This method can be applied to determine the relative number of antigenic points in the discoplasm for agglutinogens of different blood group systems. It is also applied in babies with erythroblastosis as an additional criterion of severity, and in patients having acquired hemolytic anemia, where it has been found that the degree of coating is correlated with the clinical manifestations. Thus a rise in the degree of coating may be associated with a hemolytic crisis, while a decrease may presage a remission. Disseminated lupus erythematosus appears to be one of the milder manifestations of auto-sensitization, and this view is supported by the results of the quantitative direct antiglobulin test.

III. Titration of Rh Antibodies in Serum and Identification of Specificity

Rh antibody titrations are made on all Rh-negative sensitized patients, and on all other bloods as ordered. The serum of Rh-positive patients sensitized against specific Rh factors is also titrated against these specific cells. For example, serum from Rh-negative patients is titrated against the following cells: (1) group O type Rh₁Rh₁ (if not available, use type Rh₁rh); (2) group O type Rh₂rh; (3) group O type rh'rh; (4) group O type rh''rh; and (5) group O type rh (triple Rh negative).

¹Coombs, R. R. A., Mourant, A. E., and Race, R. R.: *Brit. J. Exper. Path.* 26: 255, 1945.

²Wiener, A. S., and Gordon, E. B.: *Am. J. Clin. Path.* 23: 5, 1953.

If the patient lacks **Rh₀** but has **rh'**, the blood is not titrated against the **rh'**. If the patient is sensitized against the **Hr** factors, the serum is titrated against Rh-negative blood, and Hr-negative blood is used as a control instead of Rh-negative (Rh-negative blood is Hr-positive).

Six titrations are run simultaneously:

- (1) Saline agglutination titer for bivalent antibodies.
- (2) Trypsinated cell titer.
- (3) Indirect blocking antibody titer, for univalent antibodies.
- (4) Antihuman globulin titer, for univalent antibodies.
- (5) Albumin-plasma titer, for univalent antibodies.
- (6) Trypsinated cell-antihuman globulin titer, for univalent antibodies.

The serial dilutions which are used are: 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512. Additional dilutions are made if the titer is 1:512 or higher.

If the sensitivity tests which were made before the titration indicate that the antibody titer will be weak, some of the tubes may be omitted.

Three racks of 10 holes and 4 rows are needed for the titration proper (two for saline-suspended cells, and one for trypsinated cells), and one rack is used to make the serum dilutions. A rack with 2 rows of holes may be used to make the dilutions. The technic of handling dilutions is extremely important; no traces of serum or of the various dilutions must be carried from one tube to another, as that would erroneously increase the titer.

The cells needed are listed above. One set must be untreated and suspended in saline to a 2 or 3 per cent suspension; they are washed once before being suspended. Trypsinated cells are prepared as usual (see page 1006), and resuspended to a 2 or 3 per cent in saline.

Albumin-Plasma Mixture.—

Pool a number of groups O, A, and B plasmas.

Centrifuge and remove the clear plasma.

Measure 8 drops of 30% bovine albumin into a tube.

Rinse the dropper with the pooled plasma, discarding each dropperful of rinsing plasma.

Do this 2 or 3 times depending on how much plasma there is. This is to remove the bovine albumin from the dropper without admitting any water or saline.

Using the same dropper, add 32 drops of the pooled plasma, so that there is 1 part of the albumin to each 4 parts of pooled plasma.

Mix the albumin-plasma mixture in and out of the dropper before using.

For the indirect blocking test, anti-**Rh₀** saline agglutinating serum will be needed.

When the titrations have been prepared, the first row of tubes in the titration racks will contain serially diluted serum plus group O **Rh₁Rh₁** cells; the second row, the serially diluted serum plus group O **Rh₂rh** cells; the third row, group O **rh'rh** cells (5 tubes unless a high titer is anticipated); and the fourth row, the first two tubes will contain serially diluted serum and group O Rh-negative cells, and serially diluted serum plus group O **rh''rh** cells in the second half of the fourth row.

In rack 2, controls are run on the various cells suspended in saline, plus the albumin-plasma mixture, to be sure that these cells do not agglutinate in the albumin-plasma mixture without the patient's serum.

One drop of each serum dilution is used for each drop of cells.

Actual Technic of the Titration (see Fig. 254).—

Dilutions and titrations are made at the same time so that the same dropper can be used throughout the procedure, insuring uniform drops in each tube.

Rack up the three racks of tubes, 7.5 cm. by 8 mm., for the titrations. Be sure to label at least the first tube in each row. Use a different color wax pencil for the trypsinized cells titrations. When antiglobulin tests are carried out, each tube must be labeled.

Rack up a set of Kahn tubes in two rows of 10 tubes each to make the dilutions of serum.

Use serum, NOT plasma, for the titration whenever possible.

Place the serum in tube 1 of the front row of the dilution rack. Leave the other 9 tubes in that row empty. Label the tubes 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512, respectively.

Fill all the back row of dilution tubes with saline. There are now two rows of Kahn tubes, the first row empty except for serum in the first tube, and the second row of tubes filled with saline.

Put 1 drop of the undiluted serum into the first tube of each row in the titration.

Using the same dropper, carry 20 drops from the first tube of serum to the second tube in the front row of the dilution rack.

Rinse the dropper with all the saline in tube 1 of the back row of dilution tubes, and remove the discarded saline tube from the rack. Wipe off the excess saline with a gauze pad.

Put 20 drops of clean saline into each tube of the dilution rack, front row, beginning with tube 2, and using the same dropper which has been rinsed with saline.

Mix the contents of tube 2 thoroughly in and out of the dropper, avoiding bubbles.

Transfer a drop of this dilution into each second tube of the titrations, holding the pipette at the same angle throughout, and being careful not to add bubbles, for these are not full drops.

Carry 20 drops of diluted serum from tube 2 to tube 3 of the dilution rack.

Rinse the dropper with the saline in the tube behind tube 2, using all the saline, and wiping off the excess on a gauze pad.

Mix contents of tube 3 thoroughly, in and out of this same dropper, carry a drop to each tube 3 in the titrations, and carry 20 drops to tube 4 in the dilution rack.

Rinse the dropper with the saline behind tube 3, and continue in the same manner as above, until all dilutions have been made, and all the tubes in the titration racks have received their respective diluted serum, one drop each.

Do not put any of the diluted serum in the control tubes that are run on the cell suspensions which are to receive albumin-plasma mixture.

Adding the Untreated Saline-Suspended Cells.—

Add 1 drop of untreated saline-suspended group O Rh₁Rh₁ cells to each tube in the front row of racks 1 and 2.

Add 1 drop of group O Rh₂rh untreated saline-suspended cells to each tube of the second row in racks 1 and 2.

Add 1 drop of group O rh'rh cells untreated and saline suspended into each tube of the third row of racks 1 and 2.

Add 1 drop of group O Rh-negative cells, untreated and saline suspended, into the tubes marked for these cells, the first two or three in row 4 of the titration racks 1 and 2. If full titrations are run, there will be 10, not 2 or 3, of these tubes.

Add 1 drop of group O rh''rh untreated saline-suspended cells to each of the tubes marked for these cells (in the right-hand side of row 4 of racks 1 and 2 of the titrations). Full titrations may be run on these if desired, requiring 10 tubes, which would mean 5 rows of tubes for racks 1, 2, and 3, instead of 4 rows.

Prepare the control cell suspension tubes in rack 2, or else use a separate rack. Add 1 drop of each cell type to its respective tube. These will not receive any serum.

Preparation for Trypsinated Cell Titrations.—

In rack 3 for the titrations, add 1 drop of group O type Rh₁Rh₁ trypsinated cells to each of the front row of tubes.

Add 1 drop of group O type Rh₂rh trypsinated cells to the second row of tubes.

Add 1 drop of group O type rh'rh trypsinated cells to the third row of tubes.

Add 1 drop of group O type rh"rh trypsinated cells to the fourth row of tubes.

Add 1 drop of group O type rh trypsinated cells in the tubes marked for these cells in row 4.

Incubate all three racks, after mixing each tube, for 1 hour in a water bath at 37° C. The bath should be covered with a white cloth to keep dirt out of the tubes.

At the end of the hour, take bottom readings on the tubes, but do not disturb rack 2, which is exactly like rack 1 up to this point (except for the controls). It will be used later for the albumin-plasma mixture. It must stand undisturbed.

I. Saline Agglutination Titer.—

Read the result in each tube of **rack 1** under a scanning lens of a microscope, tilting the tube back and forth to resuspend the cells gently. The weakest dilution with a positive reaction is the titer. This gives the **saline agglutinating titer**.

II. Albumin-Plasma Titer (Wiener Test).—

Using a dropper with a fine capillary, remove all the saline from each tube in rack 2, beginning with the weakest dilution and working back to the strongest. Do not introduce any serum into the control tubes, which must be handled first. In other words, remove the saline from the control tubes, then begin with tubes 10 and remove the saline from each tube 10, then from each tube 9, then each tube 8, etc., down to tube 1.

Prepare the albumin-plasma mixture as directed above, and while the dropper (this is a second dropper) contains the mixture, add 1 large drop of the mixture into each tube in rack 2, including the controls.

Shake the rack thoroughly. Incubate at 37° C. in a water bath for 1 hour.

At the end of the hour, read under a scanning lens of the microscope, after looking at the bottoms of the tubes. Record clumping as positive and no clumping as negative.

Always read the controls first, and use the Rh-negative tubes for comparison when not sure of a negative reading.

The weakest dilution with a positive reaction is the **albumin-plasma titer**.

III. Indirect Blocking Antibody Titer (Wiener Test).—

Using rack 1, remove the saline from each tube in the front row of tubes where there is a negative reaction. If the reaction is positive, the test is not run. This test is for the "indirect blocking" reaction. Only the first row of tubes is used.

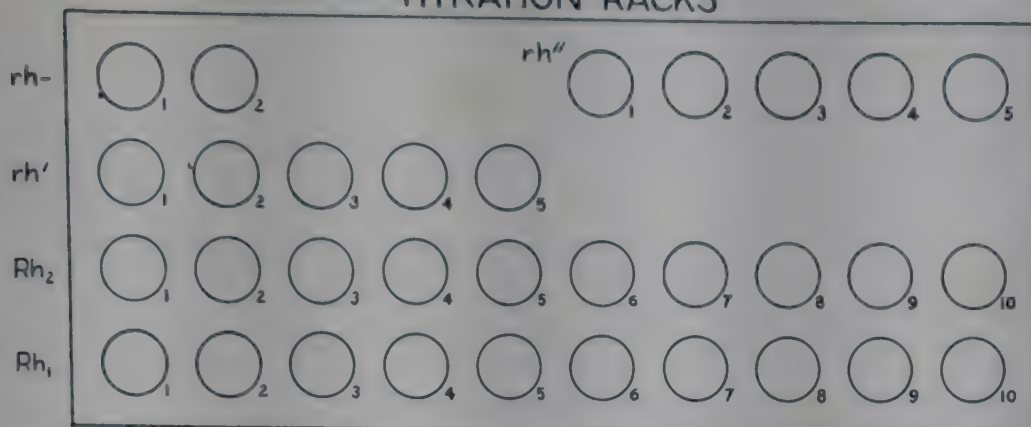
Add 1 drop of anti-Rh₀ saline agglutinating serum to each tube which shows a negative reaction, and *only to row 1 of the first rack*. This detects the Rh₀ blocking antibody.

Shake and replace the tubes in a water bath at 37° C. for 1 hour.

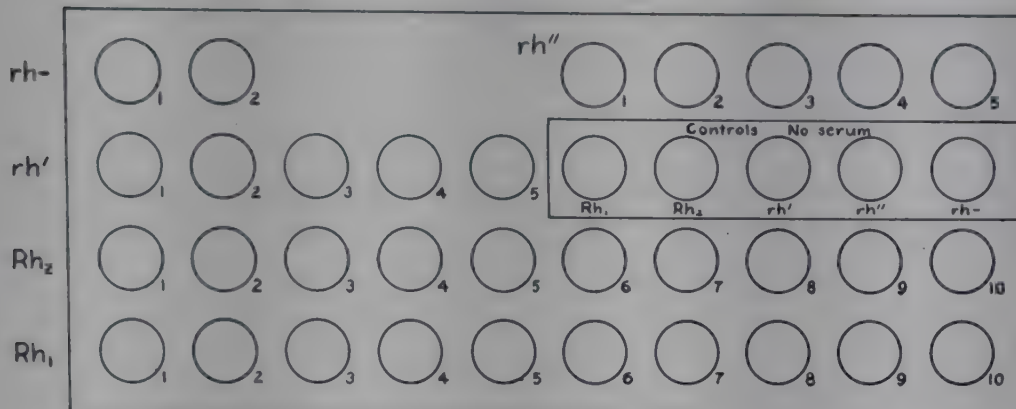
At the end of the hour, read under a scanning lens, and record. The last tube showing a **NEGATIVE** reaction is the titer. Explanation: In the first hour, the patient's serum was mixed with known Rh-positive cells. If these cells did not agglutinate, either the patient's serum did *not* contain bivalent (saline-agglutinating) antibodies, or else it *did* contain univalent (blocking) antibodies, which simply coated the cells but did not agglutinate them. Known anti-Rh₀ agglutinating serum was then added, which has the ability to agglutinate Rh-positive cells. Since these Rh-positive cells did not agglutinate, they must have been coated with blocking antibody derived from the patient's serum, which prevented their clumping when the known anti-Rh₀ serum was added. If they had clumped, that would indicate that there were no blocking antibodies in the patient's serum.

This gives the **indirect blocking titer**.

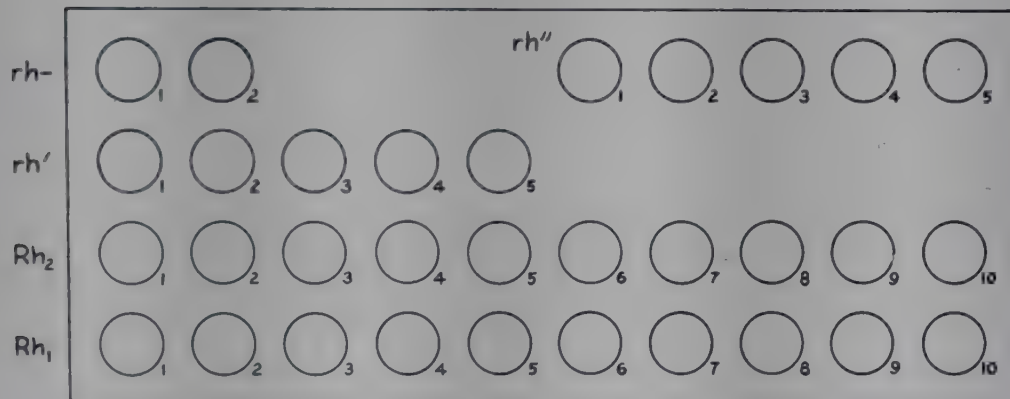
SET-UP FOR FULL TITRATIONS FOR Rh ANTIBODIES TITRATION RACKS



For trypsinated cells

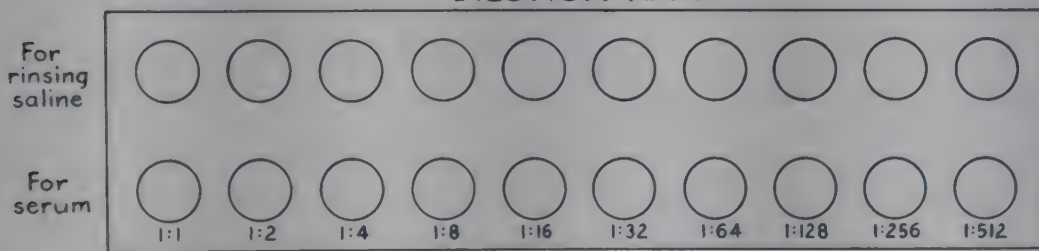


For untreated saline suspended cells



For untreated saline suspended cells

DILUTION RACK



For dilutions

Undiluted serum here and 1 drop in each tube 1 of titrations. Carry 20 drops to tube 1:2	Mix. Carry 1 drop to each tube 2 and 20 drops to 1:4	Mix. Carry 1 drop to each tube 3 and 20 drops to 1:8	Mix. Carry 1 drop to each tube 4 and 20 drops to 1:16	Mix. Carry 1 drop to each tube 5 and 20 drops to 1:32	Mix. Carry 1 drop to each tube 6 and 20 drops to 1:64	Mix. Carry 1 drop to each tube 7 and 20 drops to 1:128	Mix. Carry 1 drop to each tube 8 and 20 drops to 1:256	Mix. Carry 1 drop to each tube 9 and 20 drops to 1:512	Mix. Carry 1 drop to each tube 10. Save for further dilutions if necessary.
Rinse dropper and put 20 drops saline in the rest of the tubes in front row.	Rinse dropper	Rinse dropper	Rinse dropper	Rinse dropper	Rinse dropper	Rinse dropper	Rinse dropper	Rinse dropper	

Fig. 254.

IV. Anti-Human Globulin Titer (Coombs Test).—

Using rack 1, add saline to each negative tube in rows 2, 3, and 4, after shaking. Fill the tubes about $\frac{3}{4}$ full.

Centrifuge and pour off the supernatant fluid, catching the last drop on a gauze pad.

Shake each tube, add saline again, and again centrifuge. This is the second washing. Remove the saline, replace with fresh saline, centrifuge, and remove saline, this time tapping the tubes hard against a gauze pad to remove all traces of saline. Take care not to lose the cells. This is the third washing.

Shake the tubes, and add a drop of Coombs serum (antiglobulin) to each tube.

Centrifuge for 2 minutes at 500 to 1,000 r.p.m. without incubation.

Read all tubes under a scanning lens of a microscope, and report clumping as positive and lack of clumping as negative. Read the controls first for comparison. The weakest dilution with a positive reaction is the **antiglobulin titer**.

This completes the tests on saline-suspended untreated cells.

V. Trypsinated Cell Titer (Morton and Pickles).—

At the end of the hour's incubation of rack 3 containing the trypsinated cells, read under a scanning lens. These tubes are handled not quite so gently as the untreated cells. Read the negative reactions first for comparison, then read the other tubes. Record clumping as positive and lack of clumping as negative. The weakest dilution with a positive reaction is the **trypsinated cell titer**.

VI. Trypsinated Cell-Antiglobulin Titer (Unger Test).—

Use all the tubes in rack 3 which give a *negative* reaction.

Wash 3 times with saline as directed above in the other tests.

After the last washing and packing, remove all the saline by tapping hard against a gauze pad, resuspend the cells, and add a drop of Coombs serum to each tube.

Centrifuge for 2 minutes at 500 to 1,000 r.p.m. and read the reactions under a scanning lens. The weakest dilution with a positive reaction is the **trypsinated cell-antiglobulin titer**.

Reactions are recorded by Unger as ++s, ++, ++w, +s, +, +w, \pm , and -.

++s means a solid clump which does not break up when being handled.

++ means not quite so solid a clump.

++w means the clump breaks up partially while handling.

The other reactions are gradations of this reaction.

Be very careful not to read false clumping due to settling of cells, or to rouleaux formation.

All trypsinated cell tubes will at first give the impression of a positive reaction, but a true positive reaction does not entirely disappear during handling. The weak ones might be difficult to read, but if they stand for a while at room temperature, the clumping returns.

The titer is the reciprocal of the last tube in which a positive reaction occurs (except in the indirect blocking reaction, where the titer is the reciprocal of the last negative reaction). See example, page 1071.

On all tubes where two tests are made, for example a Coombs test made after the saline agglutination test is negative, or an indirect blocking test after the saline test is negative, the results are recorded by Unger as follows:

- | This means the first test was negative but the second test was also negative (top result in the box).
- +| This means that the first test was negative, but the second test (antiglobulin, etc.) was positive.

NOTE: For the indirect blocking result, the box opens to the right: $\begin{array}{|c} + \\ - \end{array}$

For the antiglobulin test, it opens to the left: $\begin{array}{c} + \\ - \end{array}$

Examples: See Tables 117 A, 117 B, and 118.

TABLE 117 A.—RACK 1: SALINE-AGGLUTINATING, INDIRECT BLOCKING, AND ANTIGLOBULIN TITER

	1	2	4	8	16	32	64	128	256	512	
Rh ₁ Rh ₁	<u>++++s</u>	<u>++++s</u>	<u>++++s</u>	<u>++++s</u>	<u>++++s</u>	<u>++++s</u>	<u>++++s</u>	<u>++++s</u>	<u>++++s</u>	<u>++++s</u>	No titer
Rh ₂ rh	<u>++++s</u>	<u>++++s</u>	<u>++w</u>	<u>++w</u>	<u>+s</u>	<u>+s</u>	<u>+w</u>	<u>-</u>	<u>-</u>	<u>-</u>	64
rh'rh	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	0
rh''rh	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	0
rh	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	0

TABLE 117 B.—RACK 2: ALBUMIN-PLASMA TITER

	1	2	4	8	16	32	64	128	256	512	
O Rh ₁ Rh ₁	<u>++s</u>	<u>++s</u>	<u>++s</u>	<u>++w</u>	<u>++w</u>	<u>+s</u>	<u>+</u>	<u>±</u>	<u>-</u>	<u>-</u>	64
O Rh ₂ rh	<u>++</u>	<u>++</u>	<u>++</u>	<u>++w</u>	<u>+s</u>	<u>+s</u>	<u>+w</u>	<u>+w</u>	<u>-</u>	<u>-</u>	128
O rh'rh	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	0
O rh''rh	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	0
O rh	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	0

TABLE 118.—RACK 3: TRYPSINATED AND TRYPSINATED-ANTIGLOBULIN TITER

	1	2	4	8	16	32	64	128	256	512	
O Rh ₁ Rh ₁	<u>++s</u>	<u>++s</u>	<u>++s</u>	<u>++</u>	<u>++</u>	<u>++w</u>	<u>+</u>	<u>+w</u>	<u>+</u>	<u>-</u>	128-256
O Rh ₂ rh	<u>+++</u>	<u>+++</u>	<u>+++</u>	<u>++s</u>	<u>++s</u>	<u>++</u>	<u>++</u>	<u>+s</u>	<u>+</u>	<u>-</u>	128-256
O rh'rh	<u>++w</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	1
O rh''rh	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	0
O rh	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	0

On the indirect blocking, the last **NEGATIVE** is the titer. If O Rh₁Rh₁ tubes are negative, remove saline and add anti-Rh₀ tube serum. On other negative tubes, do the Coombs test.

When negative, do the Coombs test after washing 3 times with saline.
In this example, Figs. 117A, 117B, and 118, the titers are as follows:

- 1. Indirect blocking: zero units.
- 2. Saline agglutinating: zero units.
- 3. Antiglobulin, 64 units against Rh₁Rh₁ and Rh₂rh.
- 4. Albumin-plasma method, 64 units against Rh₁Rh₁ and 128 units against Rh₂rh.
- 5. Trypsinated cell method, 128 units against Rh₁Rh₁ and also against Rh₂rh, and 1 unit against rh'.
- 6. Trypsinated-antiglobulin method, 256 units.

NOTE: If the serum gives a positive reaction against Rh-negative cells, there is an anti-hr' or anti-hr'' antibody present, or some mistake has been made.

Simplified Titration of Rh Antibodies in Serum
For Routine Hospital Use

Although the foregoing method of titration and determination of specificity gives valuable information, routinely it is not necessary to conduct all

six of the titrations. Three should suffice: the saline agglutination titration method, the antihuman globulin titration method, and the trypsin-treated cell titration method.

When Rh-negative persons are sensitized by an Rh factor, the specificity of the antibody usually involved is anti-Rh₀. Often anti-rh' is also present, and far less frequently anti-rh''. For this reason, it will suffice, for routine practice, to use untreated saline-suspended group O type Rh_zRh₀ cells and trypsinated saline-suspended group O type Rh_zRh₀ cells. Thus there will be only two rows of tubes for titrations, and, in addition, the Rh-negative controls.

Make serial dilutions as directed under the method given above: 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512.

While making the dilutions place 1 drop of each dilution in its respective tube of three rows of tubes.

Add 1 drop of untreated saline-suspended group O type Rh_zRh₀ cells to each tube in the first row, and 1 drop of group O type Rh_zRh₀ trypsinated cells to each tube of the second row. In the third row, which should have 10 tubes (5 dilutions each for the negative controls), add 1 drop of the untreated saline-suspended group O rh cells to the first 5 tubes and 1 drop of trypsinated saline-suspended group O rh cells to the second 5 tubes (1:1, 1:2, 1:4, 1:8, 1:16).

Incubate all tubes for 1 hour at 37° C. in a water bath.

Read the bottoms of the tubes, and also read the reactions under a scanning lens.

Carry out antiglobulin tests on all negative reactions. Wash three times with saline. Remove all traces of saline after the last washing, using the technic outlined above.

Shake each tube and add 1 drop of Coombs serum (antihuman globulin) to each.

Centrifuge at 500 to 1,000 r.p.m. for 2 minutes, and read.

The weakest dilution giving a positive reaction is the titer. See the method above for recording the results.

Preparation of Anti-Rh Serum for Testing Purposes*

From Normal Male Donors

Owing to the difficulty of securing an adequate amount of anti-Rh serum from iso-immunized women, Wiener and Gordon¹ have suggested the manufacture of this serum by injecting normal male professional blood donors. Their theory was based upon the fact that injections of relatively minute amounts of solutions A and B group substances into individuals lacking the corresponding agglutinogens stimulated a pronounced rise in the α and β agglutinin titers in such individuals.

Their method of immunization for the preparation of anti-Rh agglutinating serum is as follows: Group O, Rh-positive blood is mixed with an equal volume of sterile citrate solution. The blood used in all experimental work belonged to type Rh_zRh_z. Four c.c. of this 50 per cent blood suspension were injected intravenously into each of a number of donors. After an interval of four months (the maximum survival time for transfused red blood cells) a second intravenous injection was given, and ten days later blood was drawn for testing. Donors with a satisfactory titer of anti-Rh agglutinins were bled.

Their original work comprised the injection of nine donors. Of these, four showed no evidence of antibody formation while five had anti-Rh agglutinins in their sera. In two donors the titers of anti-Rh agglutinins were very high, 200 and 600 units, respectively. By the method of exchange transfusion with type rh donors, 1500 c.c. of blood were drawn from each man and a satisfactory yield of testing serum was obtained.

For more recent statistics, see the Rh-Hr Types, pages 1034 ff.

*It is best to purchase these sera from U. S. Government licensed manufacturers.

¹Wiener, A. S., and Gordon, E. B. S.: *Am. J. Clin. Path.* 17: 67-70, 1947.

Erythroblastosis Fetalis

Erythroblastosis fetalis, as stated elsewhere, may occur as a result of iso-immunization of an Rh-negative pregnant woman mated to an Rh-positive man, when the fetus is Rh-positive. Much more rarely other blood factors, Hr, Kell, etc., may produce the same effect if present in the blood of the fetus and absent from that of the mother. Two types of antibodies may be formed by the mother, "univalent,"* which are capable of passing through the placenta, and "bivalent,"* which are not. Univalent Rh antibodies, when they pass through the placenta, combine with and coat fetal blood cells for which the antibodies are specific.

The principal factor in determining the severity of the disease is the mother's antibody titer, because it in turn determines the degree of coating of the fetal cells. It must be understood that simple coating of the fetal red cells need not necessarily interfere with their function; not only must they be coated, but a third component, or "X" protein (conglutinin), must be present before clumping takes place. Although complement and complement-like substances (conglutinin or X protein) are not fully developed before birth, if the mother's antibody titer has been high for sufficient time the child may be stillborn, or, if born alive, may manifest symptoms of severe erythroblastosis fetalis. Often the baby appears normal at birth, but early in the neonatal period symptoms appear.

Treatment of the sensitized Rh-negative mother with so-called "Rh happen," diethylenesulphonate, progesterone, exchange transfusions of the mother, cortisone, "desensitizing" injections of Rh-positive blood have been unsuccessful. The only treatment which has been successful to date is the so-called "exchange" transfusion of the baby at birth before the baby's cells have had a chance to become clumped or hemolyzed (see pages 1075 ff.). In this transfusion, the baby's Rh-positive erythrocytes, as well as some plasma containing Rh univalent antibodies derived from the mother, are removed while being replaced with Rh-negative erythrocytes which cannot be coated by the offending antibodies.

The disease is characterized by abnormal destruction and regeneration of erythrocytes. It is fair to assume that intravascular clumping initiates all the other pathologic changes in this condition. Much of the pathology depends upon an excessive destruction of erythrocytes and upon an attempt on the part of the body to compensate for this loss. Anemia, increased bilirubin in the blood and in various fixed tissues of the body, abnormal erythropoiesis in many organs, including the spleen and liver, and an increase in immature forms of erythrocytes in the circulating blood are among the signs most commonly observed. (For a description of the blood picture in erythroblastosis fetalis, see page 981.) The extreme edema present in some of these cases has not as yet been explained. Some have explained it on the basis of renal failure, injury of the general capillary bed, and a decrease in plasma proteins occurring in conjunction with cellular hemolysis.

*The terms univalent and bivalent are not to be taken literally in the chemical sense but are convenient terms for designating the two forms of antibodies and for visualizing their behavior in vitro and in vivo.

There is no direct relationship between the depth of jaundice and the degree of anemia. The jaundice is hepatogenous as well as hemolytic in origin. In addition, although brain damage occurs more often in severely jaundiced babies, some severely jaundiced babies may recover completely and some with lighter jaundice die with brain damage. Wiener* believes that the tissue damage is due primarily to vascular injury resulting from intravascular conglutination of the baby's coated cells. Damage to the liver results in jaundice. Blockage of circulation in the brain produces nuclear damage and allows bilirubin to cross the blood-brain barrier and stain the dead and dying ganglion cells. Therefore, if the coated Rh-positive cells are removed before intravascular clumping occurs, and are replaced by Rh-negative cells which will not combine with the free Rh antibody, tissue damage may be prevented. Tissue damage which may be present at birth appears to be largely reversible. Kernicterus is rarely if ever found in newborn or stillborn infants. This is to some degree probably due to the fact that bilirubin formed in utero passes into the maternal circulation and is largely excreted. The rapidly developing jaundice and organic injury are probably due to increase in the concentration of complement or conglutinin in the infant's circulation during the early neonatal period. This results in intravascular clumping and hemolysis.

Sensitization to the A-B-O Factors

Halbrecht,¹ Polayes and McNally,² Wiener, Sonn, and Hurst³ called attention to cases of A-B-O sensitization, in which the Rh factor had no part whatsoever. Mild cases of erythroblastosis due to A-B-O incompatibility appear to be quite common but are usually overlooked. Most infants recover spontaneously, but in some cases the anemia is severe enough to warrant transfusion.

It was noticed first by Rosenfield⁴ that erythroblastosis due to an A-B-O incompatibility is almost always confined to group O mothers. This can readily be seen in earlier reports on series of cases by Wiener, Sonn, and Hurst, and by Wiener, Wexler, and Hurst. Wiener and Unger showed that group O babies of group O mothers acquire by placental transfer about 16 times as much isoantibody as babies having mothers of group A or group B. They concluded that this was adequate to account for the excess of group O mothers with babies having A-B-O hemolytic disease.

As explained elsewhere in this chapter (page 1000), agglutinogens A and B are both characterized by factor **C**, so that there is no anti-**C** present in the sera of groups A, B, and AB. Group O erythrocytes are the only ones which do not contain the **C** factor, and therefore, group O serum is the only variety which can contain antibodies specific for factor **C**. These antibodies, Unger and Wiener⁵ showed, are of the univalent variety, and traverse the placental barrier. Thus if the mother's blood is group O and that of the fetus is group

*Exper. Med. & Surg. 7: 200, 1949.

¹Halbrecht, I.: Am. J. Dis. Child, 69: 208, 1944.

²Polayes, S. H., and McNally, I., Jr.: Am. J. Clin. Path. 18: 375, 1948.

³Wiener, A. S., Sonn, E. B., and Hurst, J. G., Studies on Individual Differences in Human Blood. Paper No. 1, July 15, 1946, Brooklyn, N. Y.

⁴Rosenfield, R., A-B-O Hemolytic Disease. Proc. Am. Assoc. Blood Banks, 1953: cf. Wiener, A. S., Sarnwick, A. A., Morrison, H., and Cohen, L. Exper. Med. and Surg. 11: 276, 1953.

⁵Unger, L. J., and Wiener, A. S.: J. Lab. & Clin. Med. 44: 3, 1954.

A, the fetal erythrocytes contain the **C** factor, whereas the maternal erythrocytes lack it, and anti-**C** may be formed in excess by the mother. These antibodies, in turn, traverse the placenta and enter the fetal circulation. Since these **C** antibodies cross-react with both A and B agglutinogens, if these are present in the baby's plasma in sufficient amount they may produce hemolytic disease in infants. This entire question has been discussed in detail by Unger and Wiener.⁵

In reference to these cases, Wiener⁶ concluded, first, that the A-B-O blood properties appear to be the most common cause of erythroblastosis in infants with Rh-positive mothers; second, the natural anti-A and anti-B agglutinins in maternal sera, as a rule, have little or no harmful effect on fetuses belonging to groups A, B, and AB, because they usually are of low titer and avidity and because the natural isoagglutinins presumably are larger molecular aggregates which do not traverse the placenta easily; third, individuals may become iso-sensitized against the A and B properties by the inadvertent transfusion of blood of an incompatible group, from pregnancy with a fetus of an incompatible group, or by injections of materials containing soluble group substances such as pooled human serum, therapeutic horse serum, etc.; fourth, immune isoantibodies for A and B in the maternal serum (anti-**C**) are more dangerous for the fetus than the natural isoagglutinins because they are more likely to be of high titer and, in addition, pass through the placenta more readily; fifth, cases of congenital hemolytic disease due to A-B-O sensitization are usually milder than cases due to Rh sensitization and patients frequently recover without treatment, but severe or even fatal cases, with liver damage and kernicterus, also occur and in these cases the A-B-O factors are the cause; sixth, when Rh-negative individuals are exposed to Rh-positive blood of an incompatible blood group, the principle of competition of antigens may operate, so that the agglutinogens A or B may suppress the antigenicity of the Rh factor; and seventh, in two of Wiener's cases failure to consider the possibility of A-B-O sensitization caused confusion in diagnosis, because Rh-negative mothers had Rh-positive erythroblastotic infants, yet the maternal sera did not contain Rh antibodies.

In case of pregnancy in a union between Rh-positive husband and Rh-negative wife, one may expect the first-born to be free from symptoms. Physicians should be ready to proceed with the proper treatment of children of an Rh-positive father and an Rh-negative mother by exchange transfusion with Rh-negative blood.

A-B-O hemolytic disease, in contrast to that caused by the Rh factor, frequently affects the first-born infant.

The Treatment of Erythroblastosis Fetalis by Exchange Transfusions

Wiener, Wexler, and Gamrin⁷ in 1944, adopted a method of treating erythroblastosis fetalis by exsanguination transfusion, or "exchange transfusion." This is a method of simultaneously removing the infant's blood and replacing it with other blood. In the case of Rh sensitization, Rh-negative

⁵Wiener, A. S., Wexler, I. B., and Hurst, J. G.: *Blood* 4: 1014, 1949.

⁷Wiener, A. S., Wexler, I. B., and Gamrin, E.: *Am. J. Dis. Child.* 68: 317, 1944; Wiener, A. S., and Wexler, I. B.: *J. Lab. & Clin. Med.* 31: 1016, 1946.

blood is introduced into the infant's circulation while at the same time removing the Rh-positive baby's blood containing Rh isoantibodies derived from the Rh-negative sensitized mother. The same principles apply in the cases of A-B-O hemolytic disease and isosensitization to Hr, Kell, and other blood factors.

The rationale of exchange transfusion is explained on pages 1073 and 1074. It has been known for some time that anemia is not the only cause of death in erythroblastosis, and in fact is an infrequent cause. Many babies who have been treated adequately by simple blood transfusions and even babies who have never developed any anemia rapidly have become more and more deeply jaundiced and have died on the second to fifth day of life with pathologic evidence of kernicterus, and often with liver necrosis and intrapulmonary hemorrhage. Wiener has pointed out that the immediate noxious agent in such cases is the baby's coated red cells.

There is no direct relationship between the depth of jaundice and the degree of anemia in this disease; therefore, the jaundice is not solely hemolytic in origin. Moreover, as has been pointed out above, tissue damage is due primarily to vascular injury resulting from intravascular conglutination of the baby's coated cells. Damage to the liver results in jaundice, and blockage of the circulation in the brain produces nuclear damage and allows bilirubin to enter the brain and stain the dead and dying ganglion cells. Thus it is evident that tissue damage cannot be prevented or counteracted by simple addition of blood. However, if the coated Rh-positive cells are removed before intravascular clumping occurs, and are replaced by Rh-negative blood cells which cannot be coated with the free antibody, tissue damage can be prevented.

Technic of Exchange Transfusion⁸.—

Since bleeding and administration of blood must be carried out simultaneously, total replacement of the baby's red cells cannot be attained. A pint of blood, or about twice the baby's blood volume, is used. This will accomplish 85 per cent replacement. The blood used should be less than 2 days old, and is concentrated to a hematocrit of 0.50 by removing part of the citrated plasma to the 400 c.c. mark.

Immobilize the infant on a circumcision board by the use of flannel wrappings, leaving one arm and one leg free.

Strap the leg down with adhesive tape, and expose the saphenous vein at the ankle through a transverse incision made anterior and superior to the medial malleolus.

Cannulate the vein with a blunt 20 gauge needle (a 20 gauge polyethylene catheter may also be used), and tie in place.

Inject through this needle 200 units of heparin diluted in 2 c.c. of saline.

Attach a three-way stopcock, which in turn bears the tubing from the blood reservoir and a 10 c.c. Luer-Lok syringe for the injections of the blood.

Before any injection of blood is made, isolate the radial artery through a transverse incision on the radial side of the wrist just proximal to the head of the radius. The artery can then be recognized as a moderate-sized plump vessel, the appearance and size of which are not unlike those of the saphenous vein at the ankle. There are no veins in this part of the forearm with which it may be confused. Clean the artery carefully, lift it up on

⁸Wiener, A. S., Wexler, I. B., and Brancato, G. J.: *J. Pediat.* 45: 546, 1954.

a closed mosquito clamp, and nick by placing the point of a scalpel at about the midpoint and making an angled incision through the flattened wall. The flow of blood is immediate and copious.

For convenience, collect the blood in 30 c.c. medicine glasses, and empty these into a larger graduated receptacle for final measurement. Before administering any blood at the ankle, permit 50 c.c. of the infant's blood to run out through the arterial incision.

Then commence injecting blood at a rate which keeps pace with the arterial flow, and which makes up the deficit by the time that the 90 c.c. mark is reached.

Equal quantities of blood are administered and withdrawn (about 400 c.c.), which in the average infant will effectuate an exchange of about 90%. During the procedure, administer intravenously calcium gluconate in quantities of 1 c.c. of a 10% solution for every 100 c.c. of concentrated blood given.

At the termination of the transfusion, close the incisions with fine silk sutures and apply pressure bandages. Do not ligate either the artery or the vein; it may be assumed that they recannulate in the process of healing.

Postoperatively, give the infant 100,000 units of penicillin every 8 hours for 2 days, and, if necessary, place the infant in an incubator and give continuous oxygen therapy. Feedings are routine. In instances where the procedure is repeated, use the opposite arm and leg; however, an attempt may be made to recannulate the vein (*but not the artery*) used for the first transfusion.

The small amount of heparin introduced at the beginning of the procedure facilitates the bleeding. The procedure can be carried out without heparin. Wiener and associates have never encountered any harmful effects attributable to this quantity of heparin.

The rate at which the procedure is carried out is important. The more protracted it is, the less likely it is to cause any reaction. However, it is convenient to complete the procedure in about 45 minutes; never in less than 20 minutes. At this rate, the infant can metabolize the injected citrate rapidly enough to prevent hypocalcemia. More rapid injection carries with it the danger of citrate shock.

Either freshly drawn blood or bank blood can be used, but fresh blood is safer, since the blood cells are less fragile and the danger of bacterial contamination is virtually eliminated.

If bank blood is used, it must be as fresh as possible, preferably not more than two or three days old. For concentrated blood, use sedimented bank blood. All that is necessary is to aspirate the supernatant citrated plasma down to the 400 c.c. mark. This should be done immediately before use, the bottle inverted, and a sample withdrawn, centrifuged, and examined for hemolysis by observation of the supernatant plasma. A stained film is made for examination for bacterial contamination.

When these precautions are observed, the procedure is virtually without danger. If any difficulty is encountered with infusion, numerous other sites are available.

The umbilical catheter method of Diamond is not recommended since it has been said to have caused damage to important intra-abdominal vessels with resultant thrombosis and embolism. Intra-abdominal hemorrhage, peritonitis, and splenic rupture have also been reported following its use. There is also the danger of direct injection of citrated blood into the heart chamber.

Titration of Anti-A and Anti-B Antibodies in Serum for the Detection of Isosensitization in Erythroblastosis Fetalis, Method of Unger Modified After Wiener et al.

In cases of erythroblastosis fetalis or in sensitization unexplained by the Rh-Hr factors, it is necessary at times to titrate the anti-A and anti-B antibodies. With ordinary methods of titration, it is not possible to differentiate between natural and artificially produced anti-A and anti-B antibodies. This method attempts to make the differentiation.

Acacia.—

Dissolve 20 gm. acacia (gum arabic)
and 2 gm. Na₂HPO₄ (dibasic sodium phosphate)
in 180 c.c. of distilled water.
Autoclave at 10 pounds pressure for 10 minutes.
Use the supernatant fluid.

Cells.—

Use A₁ rh cells suspended in saline for titrating anti-A in group O or group B serum.
Use B rh cells for titrating anti-B in group O or group A serum.

Prepare the cells in the usual manner, wash once with saline, and resuspend to a 2 or 3% concentration in saline. See page 1006.

Titration for Bivalent Antibodies.—

Make serial dilutions of the serum to be tested, in saline: 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512. See page 1015 for exact technic.

Add 1 drop of the opposing cells to 1 drop of each dilution of serum (see page 1015).
Carry out two titrations on group O serum, one using A₁ rh and one using B rh cells.

Incubate in a water bath at 37° C. for 1 hour.
Take bottom readings and also read under a scanning lens.
Report clumping as positive and lack of clumping as negative.
Record the result in each tube.

Acacia Conglutination Titer.—

To each negative tube, add 1 drop of the acacia solution.
Mix and incubate in a water bath at 37° C. for 1 hour.
Read as before. Report the results. This gives the acacia conglutination titer.

Titration for Univalent Antibodies.—

At the same time, run titrations on the same serum diluted 50% with Witebsky group specific substance (1:2).

Carry out the titrations exactly as directed above, and report results in the same manner.

If there is a titer in this second set of tubes, the antibodies are due to isosensitization by A-B-O factors, since ordinarily Witebsky substance neutralizes bivalent anti-A and anti-B antibodies. Apparently it does not neutralize the univalent antibodies.

Examples: For examples, see Table 119.

TABLE 119

DILUTIONS:	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	TITER
<i>Group O Serum, No Witebsky Substance Added, No Acacia</i>											
1. A ₁ Cells	++s	++s	++	++w	+s	+s	+w	—	—	—	1:64
2. B Cells	++s	++s	++s	++w	++w	+s	+s	+w	—	—	1:128
<i>Same Serum, No Witebsky Substance, Acacia-Suspended Cells</i>											
1. A ₁ Cells	++s	++	++	++w	++w	+s	+w	—	—	—	1:64
2. B Cells	++s	++s	++s	++	++	++w	+s	+w	+w	—	1:256
<i>Same Serum, Witebsky Substance Added, No Acacia</i>											
1. A ₁ Cells	—	—	—	—	—	—	—	—	—	—	No titer
2. B Cells	++s	++	++w	++w	+w	—	—	—	—	—	1:16
<i>Same Serum, Witebsky Substance Added, Acacia Suspended Cells</i>											
1. A ₁ Cells	++	++w	+s	+w	—	—	—	—	—	—	1:8
2. B Cells	++s	++	++w	++w	+s	+w	—	—	—	—	1:32

Conclusions:

- (1) There is a 1:64 anti-A titer for bivalent antibodies.
- (2) There is a titer of 1:8 for univalent anti-A antibodies when acacia is used in the titration, but no univalent antibody titer when using saline-suspended untreated cells.
- (3) The anti-B titer for bivalent antibodies is 1:128, and 1:256 when acacia (conglutinin) is used.
- (4) The univalent antibody titer is 1:16 for anti-B for untreated cells, but 1:32 when acacia is added.

At birth, anti-A and anti-B are from the mother. All anti-A and anti-B antibodies later developed by the individual are probably of heterospecific origin.⁹ The titration described here is used to differentiate between bivalent and univalent antibodies. This patient has been isosensitized against A and B factors, and has univalent antibodies in the blood. If this has occurred in unexplained erythroblastosis fetalis, this individual is sensitized to the A-B-O factors and not the Rh-Hr factors.

Summary

1. Rh₀ agglutinogens are found in 85 per cent of Caucasoids and are absent in 15 per cent. The 85 per cent are called **Rh₀**-positive and the 15 per cent, **Rh₀**-negative. About 2 per cent of all bloods contain **rh'** or **rh''** or both, without the **Rh₀** factor. Such people must be given Rh-negative blood in a transfusion, but their blood can be safely given only to Rh-positive patients.

2. The injection of Rh-positive blood into an Rh-negative person, as in blood transfusions, may stimulate Rh antibody formation which in itself is not harmful provided no further transfusions are made. In the case of Rh-negative women in the child-bearing age, this antibody formation might lead to an erythroblastotic infant if the woman is mated to an Rh-positive man.

3. Injection of one dose of Rh-positive agglutinogens into an Rh-negative person starts a chain of immune body production. If a second transfusion of Rh-positive blood is made in an Rh-negative person already immunized with one Rh-positive injection, hemolytic reaction is almost certain to occur. This is serious and at times fatal.

4. The mating of an Rh-positive man to an Rh-negative woman may produce offspring that have been subjected to changes, either death in utero or a living child that soon after birth develops erythroblastosis fetalis. This does not always occur with the first child except in cases of Rh-negative women previously isoimmunized by an Rh-positive blood transfusion.

5. Treatment of erythroblastosis fetalis by exchange transfusion at birth, using Rh-negative blood, is the proper procedure. If no Rh-negative blood is available, the washed cells of the mother may be used.

6. All patients and all donors must be subjected to a test for the Rh factors.

7. In all pregnancies, particularly where the mother is Rh negative and the father is Rh positive, sensitization tests must be made periodically of the mother's serum, and if antibodies are found, titrations must be made at frequent intervals. If the titer is rising, one must be prepared to perform an exchange transfusion immediately if the infant is born alive. See page 1063.

⁹Wiener, A. S.: J. Immunol. 66: 51, 1951.

Agammaglobulinemia

Inhibition Test for Human Gamma Globulin, Method of Wiener^{1, 2}

Agammaglobulinemia was first described as a syndrome by Bruton,³ and later elaborated by Bruton and associates.⁴ Patients with agammaglobulinemia show complete or almost complete absence of antibodies from their serum, rendering them susceptible to recurrent infections. Their serum lacks the normal blood group isoagglutinins, as well as gamma globulin as detected electrophoretically. The other serum proteins may be within normal limits.

A test for agammaglobulinemia was devised by Wiener,¹ based on the ability of antihuman globulin sera prepared in rabbits to agglutinate human red cells coated with antibodies of human origin, such as Rh antibodies and autoantibodies. Since materials containing human gamma globulin can inhibit the reactions of such antiglobulin sera, this inhibition technic has been applied to detect the presence of human gamma globulin in patient's serum. The test can be performed quantitatively² by testing a series of progressively doubled dilutions of the material in question, at the same time carrying out parallel titrations with normal human serum as a control.

The sensitivity of the inhibition test for gamma globulin is inversely related to the titer of the antiglobulin serum used. If a low-titered serum is used, the inhibition titer will be high; the higher the titer of the antiglobulin serum, the lower the gamma globulin inhibition titer. Thus if the test is performed qualitatively only, the diagnosis of agammaglobulinemia could be missed.

Principle.—

The solution to be tested for the presence of serum globulin is mixed with absorbed antihuman globulin serum (diluted to have a titer of 10 to 20 units for maximally coated Rh-positive cells) and the mixture allowed to react in a water bath at 37° C. for one hour. Then a suspension of Rh-positive cells, coated with **Rh₀** univalent antibody, is added, and the mixture allowed to stand for a second period of incubation, after which the reaction is read. Failure of clumping to occur indicates the presence of human serum globulin in the material being tested. Occurrence of clumping indicates the absence of human serum globulin.

Reagents.—

Preparation of Rh Sensitized Cells.—

Prepare a 2% suspension of **Rh₀**-positive cells in saline.

Wash once with saline solution and resuspend to 2% concentration.

Mix with an equal volume of **Rh₀** antiserum containing univalent antibodies with a titer of approximately 100 units by the albumin-plasma method (page 1069).

Allow the mixture to react in an incubator at 37° C. for 45 minutes.

Centrifuge at moderate speed.

Discard the supernatant fluid.

Wash the sedimented cells 4 times with large volumes of saline.

Resuspend sensitized cells to an approximate 2% suspension in saline.

Antiglobulin Serum.—

Antiglobulin serum used for the test can be purchased from most institutes which manufacture blood grouping sera. It is commonly referred to as "Coombs serum." It

¹Wiener, A. S., Hyman, M. A., and Handman, L.: *Proc. Soc. Exper. Biol. & Med.* 71: 96, 1949.

²Wiener, A. S.: *Am. J. Clin. Path.* 25: 595, 1955.

³Bruton, O. C.: *Pediatrics* 9: 722, 1952.

⁴Bruton, O. C., Apt. L., Gitlin, D., and Janeway, C. A.: *Am. J. Dis. Child.* 84: 632, 1952.

is absorbed according to the requirements of the NIH before being packaged. This is diluted so as to have a titer of 10 to 20 units for the Rh-sensitized cells.

Technic.—

Prepare serial dilutions in saline of the serum to be tested and of normal serum in the same manner as for titrations, as given on page 1015. Use dilutions of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:512, etc.

Place 1 drop of each dilution in its respective small Kahn tube, properly labeled.

Add 1 drop of the diluted antihuman globulin serum to each.

Incubate at 37° C. in a water bath for 45 minutes.

Add 1 drop of Rh-positive sensitized cells to each tube.

Shake the tubes and reincubate for 60 to 90 minutes in a water bath at 37° C.

Shake gently and read the reactions macroscopically over a lighted viewing box and under a scanning lens of a microscope.

Results.—

Normal human adult serum completely inhibits agglutination in dilutions as high as 1:500 on the average, depending on the titer of the antiglobulin serum.

Normal human spinal fluid in Wiener's experiments inhibited the antiglobulin serum from reacting in dilutions of approximately 1:2 or 1:4, but this was expected because normal spinal fluid contains only about 30 mg. per cent of protein in contrast to the 7 gm. of protein found in normal human serum. Saliva showed inhibition ability to a slight degree; umbilical cord serum inhibited antiglobulin serum to the same titer as adult serum. Serum from horse, ox, and rabbit did not inhibit antiglobulin serum, and rhesus monkey serum weakened its reaction.

In cases of congenital agammaglobulinemia, inhibition titers of about one-fiftieth the normal titer were obtained. This indicates the presence of only 20 to 30 mg. of gamma globulin per 100 c.c. of serum in such individuals rather than a complete absence of gamma globulin. Following treatment with gamma globulin there is a corresponding increase in the inhibition titer.

Witebsky Substance, or A and B Group Specific Substance*

The chemical nature of the A specific substance has been investigated by Landsteiner,¹ Landsteiner and Chase,² Landsteiner and Harte,³ Meyer, Smyth, and Palmer,⁴ Goebel,⁵ Freudenberg and Westphal,⁶ and several others. These investigations confirm the work of Brahn and Schiff,⁷ Freudenberg, Eichel, and Dirscherl,⁸ and Landsteiner,⁹ who first isolated an A specific carbohydrate-like substance from horse saliva in 1932. The study of the A substance is facilitated by its occurrence in relatively large amounts in commercial preparations such as pepsin and peptone.

*Witebsky substance is now available from Sharp and Dohme, Philadelphia, Pa.

¹Landsteiner, K.: *J. Exper. Med.* **63**: 185, 1936.

²Landsteiner, K., and Chase, M. W.: *J. Exper. Med.* **63**: 813, 1936.

³Landsteiner, K., and Harte, R. A.: *J. Exper. Med.* **71**: 551, 1940.

⁴Meyer, K., Smyth, E. M., and Palmer, J. W.: *J. Biol. Chem.* **119**: 73, 1937.

⁵Goebel, W. F.: *J. Exper. Med.* **68**: 221, 1938.

⁶Freudenberg, K., and Westphal, O.: *Sitzungsber. Heidelberger Akad. Wissensch., Math.-naturwissensch. Kl.*, 1938, 1 Abhandl.

⁷Brahn, B., and Schiff, F.: *Klin. Wehnschr.* **8**: 1523, 1929.

⁸Freudenberg, K., Eichel, H., and Dirscherl, W.: *Naturwissenschaften* **20**: 657, 1932.

⁹Landsteiner, K.: *Science* **76**: 351, 1932.

Witebsky and Klendshoj¹⁰ reported a study of the nature of group specific B substance. They isolated it from stomach juice of group B subjects, shown to be rich in group specific substances. This isolation was carried out by Witebsky and Klendshoj very much like that in which Goebel⁵ isolated the A substance from commercial peptone. They found this group specific B substance in human stomach juice, determined that it was carbohydrate-like, and that it was as potent as the carbohydrate-like substance group A isolated from commercial pepsin and peptone, respectively.

Witebsky, Klendshoj, and Swanson¹¹ attempted to reduce or eliminate the anti-A and anti-B agglutinins in O blood by addition of solutions of "A" and "B" specific substances. As Ottenberg¹² first pointed out, the presence of the isoagglutinins anti-A and anti-B in the donor is at times ignored because of the dilution to which the serum of the donor is subjected in the case of a blood transfusion. Usually 500 c.c. of blood are given to adults in one blood transfusion. The resulting dilution is about 1:10 to 1:15. It is obvious that isoagglutinins present in O blood having a higher titer than 1:15 might act upon the recipient's cells if these cells contain A or B isoagglutigen. It is likely, however, that group substances¹³ in the blood as well as in the tissues capable of combining with isoantibodies represent a factor which, in addition to the dilution factor, is responsible for the fact that the isoantibodies in O blood do not produce more damage than has been reported when group O is used as a "universal donor."

For the preparation of the group substance A, commercial peptone and pepsin are used since these have been found to contain large amounts of A. The source of the group substance B is not stated by Witebsky. Witebsky found that the A substance, when isolated from peptone, forms a whitish powder which readily dissolves in water. He made up a stock solution of 1:1,000 in salt solution and used this in all of his experimental work.

Goebel Technic.¹⁴—

Four hundred fifty grams of peptone (Difco neopeptone) and 180 gm. sodium acetate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$) were dissolved in 1,350 c.c. of water, and the crude fraction containing the A substance was precipitated by the addition of 3,375 c.c. of 95% alcohol. The precipitate was collected in centrifuge tubes the following day and redissolved in 270 c.c. of water. Undissolved sediment was discarded after centrifugalization. The insoluble fraction was collected again after addition of 45 gm. of sodium acetate and 675 c.c. of alcohol. This procedure was repeated a third time using 150 c.c. of water and 24 gm. of sodium acetate and precipitating with 2.5 volumes of alcohol. The precipitate was redissolved in 180 c.c. of water, and 27 gm. of sodium acetate were added. The pH of the solution was adjusted to 4.8 by the addition of 20% acetic acid. This solution was deproteinized by shaking for 16 hours with a mixture of 40 c.c. of chloroform and 8 c.c. of butyl alcohol according to Sevag's procedure. The resulting solution gave negative tests for protein with 20% trichloroacetic acid, 5% sulphosalicylic acid and saturation with picric acid. The fraction containing the A substance was recovered from this solution by precipitation with 2.5 volumes of 95% alcohol. A solution of this substance in water was dialyzed through Cellophane against successive changes of distilled water.

¹⁰Witebsky, Ernest, and Klendshoj, Niels C.: *J. Exper. Med.* 72: 663, 1940.

¹¹Witebsky, E., Klendshoj, N., and Swanson, P.: *J. Infect. Dis.* 67: 188, 1940.

¹²Ottenberg, R.: *J. Exper. Med.* 13: 425, 1911.

¹³Wiener, A. S.: *Blood Groups and Blood Transfusion*, ed. 2, Charles C Thomas, footnote p. 55.

¹⁴Goebel: *J. Exper. Med.* 68: 221, 1938.

The solutions were kept in the refrigerator during dialysis. The active fraction was finally precipitated in 10 volumes of acetone. The substance tends to remain in colloidal suspension but forms a flocculent precipitate quite readily after the addition of a few crystals of sodium chloride. The precipitate was collected on a hardened filter paper, washed with acetone, and dried over sulphuric acid.

It was found by Witebsky that the A specific substance displayed a marked inhibitory influence on the agglutination of human blood cells of group A by serum of group B. A 1:250 dilution of his stock solution completely inhibited agglutination under the particular experimental conditions chosen, while some inhibitory influence was detectable even in dilutions of one in several millions. This A specific substance when dissolved in physiologic saline solution was markedly heat stable.

He showed the lack of toxicity of this substance by experimental work on guinea pigs. In further experimental work, he showed that no agglutination of human red blood cells of group A by plasma of group O occurred when the dilution of the plasma was as much as 1:256 or more. It is believed that the addition of A substance to O bloods is harmless and simultaneously effective by reducing the titer of the anti-A agglutinin present in O blood to such an extent as to render it practically ineffective.

In a further communication, Witebsky, Klendshoj, and Swanson¹ gave specific directions for the preparation and transfusion of safe universal blood. They called attention to an amendment to the Sanitary Code of the State of New York adopted by the Public Health Council June 26, 1940, requiring group O blood to be titrated before it is used as universal blood with a high titer of isoagglutinins. Gesse² described 46 cases of hemolytic shock following the transfusion of universal blood, 20 of which caused death of the patient. On the basis of these investigations, he formulated the following three postulates: (1) Not more than 100 to 200 c.c. of universal blood should be transfused; (2) the number of erythrocytes and the hemoglobin content in the blood of the recipient must be determined. Transfusion of universal blood is permissible if the number of erythrocytes is above 2 million. (3) The titer of the donor's blood serum toward the erythrocytes of the recipient must be determined. Only the blood of a universal donor with a titer of as low as 1:8 to 1:16 can be used.

In spite of these postulates, Gesse warns against the use of the blood of a universal donor unless, in a case of real emergency, homologous blood is not available. He opposes the use of universal blood on a large scale under war-time conditions.

Witebsky discussed his work, previously reported,³ on the use of the A specific substance added to O blood to eliminate the anti-A antibody.

The addition of the isolated group specific substances A and B caused a reduction in strength, or even elimination of the antibodies anti-A and anti-B present in the so-called universal blood of group O. Universal blood conditioned by the addition of group specific substances can be given to any patient independently of the blood group to which he belongs without the neces-

¹Witebsky, E., Klendshoj, N. C., and Swanson, P.: *J. A. M. A.* **116**: 2654, 1941.

²Gesse, E. R.: *Deutsche Ztschr. f. Chir.* **245**: 371, 1953.

³Witebsky, E., Klendshoj, N., and Swanson, P.: *J. Infect. Dis.* **67**: 188, 1940.

sity of previous determination of the patient's blood group in emergencies even without cross-matching. This blood, when kept in blood banks, is available for immediate use.

Blood group specific substances A and B are now available from Sharp & Dohme, Philadelphia, Pa., who prepare this substance in 10 c.c. sterile vials. The contents of one vial (10 c.c.) are added to 500 c.c. of group O blood.

Tisdall, Garland, and Wiener¹ showed that there is definite danger in stimulating increased antibody formation in female patients potentially capable of childbearing. For this reason, one must be careful about transfusing such women with group O blood modified by group specific substances. Certainly, however, in the face of an emergency, there should be no hesitancy in using such blood. There is also danger of stimulating antibody formation against factors as yet undiscovered.

Commenting upon this entire question, these writers believe that the use of universal donor group O blood will still play an important role in transfusion work but that group O blood must have a low titer of isoagglutinins to be safe for use as universal donor blood.² Such a low titer will mean discarding about 25 to 50 per cent of group O bloods to be used for this purpose. In the presence of a large demand for universal donor blood, such a waste of material would be unwarranted. Based on observations made in their study, it was felt that the routine addition of A and B substances to group O blood would render any group O blood perfectly safe for use as universal donor blood for military purposes. The other therapeutic role of universal donor blood is its use for the immediate treatment of emergency cases of hemorrhage in civilian life. Low titer group O blood should be transfused while proper groupings and cross-matching tests are being carried out. As an additional precaution, Rh-negative blood should be used. Because of the impracticability of having two kinds of universal donor blood, one for men and one for women, they believe that low-titer group O blood destined for use as universal donor blood in civilian hospitals should be selected according to the technic outlined in their previous paper,² without the addition of A and B substances. Their conclusions in the entire matter are presented as follows:

1. As little as 25 c.c. of a group B plasma containing a high titer of isoagglutinins caused a hemolytic reaction when administered to a group A recipient.

2. A and B group specific substances will reduce a high isoagglutinin titer to low levels.

3. Experiments on the administration of plasma with high titers of anti-A agglutinins to group A recipients present definite evidence that the addition of group specific substances A and B is a safe and reliable method for preparing all group O blood for use as universal donor blood for military purposes.

4. A and B substances are capable of antibody stimulation even when in combination with the anti-A and anti-B agglutinins of group O blood. This calls for some caution in administering such material to women potentially capable of childbearing.

¹Tisdall, L. H., Garland, D. M., and Wiener, A. S.: *J. Lab. & Clin. Med.*, 31: 437, 1946.

²Tisdall, L. H., Garland, D. M., Szanto, P. B., Hand, A. M. and Bonnett, J. C.: *Am. J. Clin. Path.* 16: 193, 1946.

5. For use as universal donor blood in civilian hospitals, Rh-negative group O blood, selected for its low natural isoagglutinin titer, is somewhat preferable to random group O blood to which group substances have been added to reduce the isoagglutinin titer.

Summary of Procedures Necessary in Carrying Out a Danger-Free Test of Donor and Recipient for Blood Transfusion Purposes*

1. Use highly potent anti-**A** and anti-**B** sera which meet the requirements of the National Institutes of Health (N.I.H.). Never use pooled sera of unknown titer, and under no circumstances use leftover sera from the serology laboratory just because they are anti-**A** or anti-**B**.

2. Keep known group A and group B blood on hand for controls. These may be taken as small clots, or they may be collected in the special anticoagulant.

3. Have on hand reliable absorbed anti-**A** serum to differentiate the subgroups A_1 from A_2 , and A_1B from A_2B .

4. Have on hand anti-**Rh**₀, anti-**Rh'**₀, and anti-**Rh''**₀ slide test sera and also anti-**Rh**₀, anti-**rh'**, and anti-**rh''** (tube) saline agglutinating sera.

5. It is well to have the anti-**Hr** sera which are available.

6. Test unknown cells against anti-**A** and anti-**B** sera, running controls at the same time.

7. Test unknown sera against known A and B cells for confirmation of blood group.

8. Subgroup all A's and AB's.

9. Test for the **Rh**₀ factor in whole blood of patients.

10. Test for the various Rh factors in whole blood of donors.

11. Cross-match in test tubes the donor's cells with the patient's serum and the patient's cells with the donor's serum. If negative, do the anti-globulin test. If positive before or after the antiglobulin test, reject the donor.

12. At the same time, do sensitivity tests on patient's and donor's sera for Rh antibodies. Do not use any donor blood which contains such antibodies.

13. Group O Rh-negative donors can be used in emergencies for post-partum cases and in retransfusions, particularly if the supernatant plasma has been removed.

14. Whenever a patient's serum shows presence of Rh antibodies, titrate to determine the concentration.

15. Whenever the donor is Rh-negative by the slide test, perform the tube test.

16. In all unexplained cases of erythroblastosis fetalis, test for other blood factors and also titrate the anti-**A** and anti-**B** antibodies.

NOTE: If a PATIENT'S blood lacks **Rh**₀, consider this patient Rh negative and give only Rh-negative (type rh) blood, even though patient's blood contains factor **rh'**, **rh''**, or both.

If a DONOR'S blood lacks **Rh**₀, but contains **rh'**, **rh''**, or both, consider this donor Rh positive, and give this blood only to Rh-positive patients. Or, better, make a tube test to determine the exact Rh type.

*See pages 1008 ff.

The Choice of Donors

The choice of donors is very important. The proper group should be selected, of course, as determined by the tests just given. In addition, the donor must be in good physical health and able to stand the withdrawal of 500 c.c. of blood without injuring his health. In many communities, donors are selected, physically examined, grouped, and kept on call whenever blood transfusions are needed.

Syphilis and Blood Transfusion.—The donor must be free from acute or chronic disease, more especially must it be proved as accurately as possible that he is not suffering with active syphilis. It is necessary first to conduct a physical examination and, second, to perform a serologic test. A complement fixation test or the VDRL test may be performed. Possibly, for rapid results, the Kline test is the method of choice. In the Continental countries, the Sachs-Georgi and the Meinicke methods are very popular. These are precipitation tests similar to our Kline test in this country. It must be remembered, however, that a negative Wassermann reaction is not complete proof that the donor is free of syphilis. A positive reaction, however, is sufficient to reject a candidate for donor for blood transfusions.

There are more than sixty-eight cases on record in which syphilis has been communicated to the recipient because of the use of a syphilitic donor's blood. Wildegans¹ details a case of a transfusion on a case of hemoptysis, in a 33-year-old man who showed, ten weeks after the transfusion, widespread syphilitic skin eruption where the donor's blood Wassermann had been negative. How many other cases have occurred and have not been reported is a matter of speculation. Moritsch and Wittman² in Vienna detailed a case in which syphilis was transmitted from a donor who had a negative Wassermann test. In this case the donor was later proved to have had a primary syphilitic manifestation at the time of the transfusion, but sufficient time had not elapsed for him to show a positive Wassermann reaction. In another case reported by Bernheim, a syphilitic son, acting as donor, communicated syphilis to his father. In this case it was not thought necessary to perform a Wassermann test. Levy and Ginsburg³ reported a similar case. Feldmann reported from Moscow, U.S.S.R., an infection from transfusing the blood of a nurse into a recipient. Nineteen days before the transfusion, a negative Wassermann had been obtained on this donor. Other cases have been reported by Dufur, Brehm, and Cordiviola.

In these cases, the syphilitic infection is transmitted directly into the blood stream without the development of any primary manifestation. As a rule, the incubation period of this blood-transmitted syphilis is from four to ten weeks. It is usually followed by a vigorous and typical roseola (*Syphilis d'emblée*).

McNamara⁴ reported eleven cases of syphilitic infection in blood transfusion where a mistake had been made in the blood reports. White reported similar cases. A positive syphilis reaction should be sufficient for rejection of a candidate as donor.

¹Wildegans, Dr. Med. Hans: *Die Bluttransfusion*, 1933, Julius Springer Verlag.

²Moritsch and Wittmann: *Zehn Jahre Bluttransfusion an der 1. Chirurgischen Klinik*, Deutsche Ztschr. f. Chir. 236: 669, 1932.

³Levy and Ginsburg: *Syphilis Transmitted by Transfusion*, Am. J. Syph. 11: 447, 1927.

⁴MacNamara, W. L.: *Am. J. Syph.* 9: 470, 1925.

Professional and voluntary blood donors, who may be exposed to infection with syphilis during intervals between transfusions, should have serologic tests made for syphilis prior to every blood transfusion regardless of the frequency of the donations and the relation of the recipient. For instance, Périn and Lefèvre⁵ reported the transmission of syphilis from mother to child by blood transfusion. As pointed out by Rein, Wise, and Cukerbaum,⁶ in their statistical survey, "The Control and Prevention of Transfusion Syphilis," blood donors' promises and assertions of noninfection cannot be depended upon and should not be taken seriously. They emphasize, too, that the Kline exclusion test is so highly sensitive that when the reaction is negative it usually excludes syphilis. It has been shown by the United States Public Health Service⁷ that the more reliable flocculation tests were specific to the same degree and more sensitive than the complement fixation tests. The committee expressed the opinion that "certain tests which may be performed rapidly on blood specimens appeared to yield results comparable to those obtained with tests requiring a longer period for their performance." The above scientific survey suggested that the following procedure be made prior to all transfusions:

- A. Establish the blood group of all volunteer donors and regroup all professional donors.
- B. Determine the suitability of the donor's blood for the recipient by cross-matching.
- C. Detect the presence of syphilis serologically by means of the very sensitive and specific Kline diagnostic and exclusion flocculation tests.
- D. Detect any clinical evidence of syphilis by means of an adequate physical examination.

Later Morgan⁸ reviewed 16 cases of indubitable transfusion syphilis, Salkind⁹ 39 cases, Klauder and Butterworth¹⁰ 35 cases, and Eichenlaub and Stolar¹¹ 41 cases. Since this last communication, seven additional cases have been recorded.^{12, 13, 14, 15, 16, 17} Among the total reported cases, in at least ten instances blood was transfused from a donor who was in the incubation period of the disease, i.e., before the appearance of a primary lesion or the development of a positive serologic test, and in at least nine other instances the donor was in the sero-negative phase of the primary stage of syphilis. Another patient was infected by a donor who had recently been treated for early syphilis and who at the time of the transfusion had a negative serologic test. In these twenty transfusions routine serologic examination of the donor's blood therefore did not disclose the presence of the infection.

With the advent of blood banks, this problem has been greatly simplified, because of two reasons: (1) ample time is available for carrying out tests for syphilis prior to using the blood; and (2) blood stored at icebox temperature

⁵Périn, L., and Lefèvre, M.: *Bull. Soc. frang. dermat. et syph.* **42**: 954-957, 1935.

⁶Rein, C. R., Wise, F., and Cukerbaum, A. R.: *J. A. M. A.* **110**: 13, 1938.

⁷Cummings, H. S., and others: *Ven. Dis. Inform.* **15**: 387, 1934; *J. A. M. A.* **103**: 1705, 1934; *Ven. Dis. Inform.* **16**: 189, 1935.

⁸Morgan, H. J.: *Am. J. M. Sc.* **189**: 808, 1935.

⁹Salkind, E.: *Sang.* **10**: 997, 1936.

¹⁰Klauder, J. V., and Butterworth, Thomas: *Am. J. Syph. Gon. and Ven. Dis.* **21**: 652, 1937.

¹¹Eichenlaub, F. J., and Stolar, R.: *Pennsylvania M. J.* **42**: 1437, 1939.

¹²Averback, S. H.: *J. Mt. Sinai Hosp.* **5**: 627, 1939.

¹³McCluskie, J. A. W.: *Brit. M. J.* **1**: 264, 1939.

¹⁴Pradera, E. Soto, and Valledor, T.: *Rev. de Cien. Med.* **2**: 38, 1939.

¹⁵Ronchese, F.: *New England J. Med.* **220**: 556, 1939.

¹⁶Papadopol, S.: *Romanie med.* **18**: 15, 1940.

¹⁷Plan, H. C., and Frazier, C. N.: *Chinese M. J.* **57**: 301, 1940.

for 72 hours is possibly incapable of transmitting syphilis even though obtained from a donor whose freshly drawn blood is infectious. In this connection, Turner and Diseker¹ report their interesting experiments on the duration of infectivity of *Treponema pallidum* in citrated blood stored under conditions obtaining in blood banks. They added *T. pallidum* to citrated blood from both human beings and rabbits, and determined the infectivity of the mixture for rabbits after various periods of refrigeration. Blood from syphilitic rabbits known to be in an infectious stage was likewise tested. Blood from human beings with early syphilis was not tested because even under the most favorable conditions rabbits are infected with difficulty with such material. Results obtained with a strain of *T. pallidum* well adapted to the rabbit were regarded as being a more reliable index of survival or death of the organism than would be the results obtained with freshly isolated strains. Their experiments showed that, under the conditions obtaining in blood banks, syphilitic spirochetes probably undergo progressive deterioration during the storage period. Even when large numbers of virulent treponemata were added to citrated whole blood the mixtures were not infectious for normal rabbits after storage for three days or longer. After storage periods of one and two days, although viable organisms were still present, they were evidently considerably reduced in numbers or in virulence, since the incubation period of the lesions resulting from the inoculation of the same amounts of the stored mixture was significantly prolonged, and in some instances, the inoculated animals escaped infection. These experimental observers believe that the infectivity of syphilis treponemata in citrated whole blood rarely persists for longer than three days at refrigerator temperature.

Bloch² obtained infection with material stored for 72 hours. Survival for 96 hours was not demonstrated. Bloch's conclusions were that there is definite risk of transmitting syphilis to the recipient of blood from patients with early syphilis if the blood has been preserved for 72 hours or less in the blood bank. Though this risk probably diminishes rapidly after 96 hours, it would still be unwise to use the blood from persons with known early syphilis.

These experimental observations confirm the statements made above and should cause one to pause in listening to speculative platitudes from those uninformed in experimental data that syphilitic blood when stored is safe and can be given with impunity. The axiom that no syphilitic blood should ever be given in transfusion, whether fresh or stored, still obtains.

Professional donors should carry a book in which is inscribed the name, residence, age, result of serologic test, and the blood group. Each withdrawal of blood should be entered in this book, together with the date.

The question of just when blood can safely be taken from the same person the second time has been much discussed. The rate of hemoglobin regeneration in blood donors has been studied by Fowler and Barer. Previous investigations by Giffin and Haines,³ who studied 84 donors, many of whom had given blood for twenty or more transfusions, indicated that donations of 500 c.c. of blood at intervals of four to five weeks are not harmful to male donors but that in female donors anemia often develops under similar conditions. Martin and

¹Turner, T. B., and Diseker, T. H.: Bull. Johns Hopkins Hosp. 68: 269, 1941.

²Bloch, Oscar: Bull. Johns Hopkins Hosp. 68: 412, 1941.

³Giffin, H. Z., and Haines, S. F.: J. A. M. A. 81: 532, 1923.

Myers¹ expressed the belief that blood should not be taken more frequently than every three months. They noted, too, that hemoglobin regeneration is slower in female than in male donors. Fowler and Barer observed 200 blood donors who had given blood for 636 transfusions at the University Hospitals in Iowa City. These were mostly young people. They found the average drop in blood hemoglobin after the removal of 555 c.c. of blood was 2.3 gm. and the average time required to replace this amount of hemoglobin was 49.6 days. The longer recovery periods were associated with a greater drop in hemoglobin and could not be correlated with the initial blood hemoglobin values. With a smaller donation and a smaller drop in the blood hemoglobin, the recovery period was shorter. Hemoglobin was regenerated in men at the rate of 0.049 gm. per hundred cubic centimeters of blood per day under these conditions, and in women the increase was 0.040 gm. per day. It is their opinion that subsequent donations may be given with safety as soon as the blood hemoglobin has returned to its original level. An interval of six weeks should be allowed between blood donations provided that at that time the hemoglobin of the donor has returned to normal. They found that the administration of 1 gm. of iron and ammonium citrates per day increased the daily hemoglobin regeneration by 49 per cent and shortened the recovery period from 49.6 to 35.2 days during the first period of its administration. It had progressively less effect after subsequent blood donations.

WHOLE BLOOD TRANSFUSION

The principal indication for transfusion of whole blood is sudden loss of blood from hemorrhage. Such cases call for immediate transfusion of whole blood.

In addition to acute loss of blood, consideration must be given to the hemorrhagic states which call for transfusions. There are two significant factors in the body's defense against hemorrhage:² (1) coagulability of the blood and (2) vascular response. Among the conditions representing defective coagulating mechanism are hypoprothrombinemia, thrombocytopenia, hemophilia, and fibrinogenopenia. Refer to pages 1114 to 1121 for discussion of the use of plasma in peripheral circulatory failure or shock. The preoperative preparation and postoperative care of patients call for transfusions when there is impaired nutrition, secondary anemia, dehydration, and lowered resistance. Transfusions for these purposes are done almost routinely in many institutions as a preoperative procedure. In hypoproteinemias, the use of plasma is preferred over whole blood or, better, an initial injection of plasma followed by an injection of whole blood.

Splendid results in whole blood transfusion during World War II have been outlined by Agress.³ As Chief of the Laboratory Service of the 21st General Hospital (Unit from Washington University, serving in North Africa, Italy, and France), Agress was in a very fortunate position to observe the effects of whole blood transfusion in the wounded in order to put them in the

¹Martin, J. W., and Myers, J. T.: *J. Lab. & Clin. Med.* 20: 593, 1935.

²Kilduffe, R. A., and DeBakey, Michael: *The Blood Bank and the Technique and Therapeutics of Transfusions*, St. Louis, 1942, The C. V. Mosby Co.

³Agress, Harry: *Washington Univ. Med. Alumni Quart.*, Jan., 1946.

proper condition for operation and rapid convalescence. He found that the use of the copper sulphate technic of Phillips, Van Slyke, and associates for determining hemoglobin, hematocrit, and total proteins lent itself to measuring the blood status of the patients. It was found by this test that many of these patients had low blood values on admission to the hospital. Of 4,404 patients, 64 per cent showed a deficit in either hemoglobin or total blood proteins, or both. The main deficiency was in hemoglobin. Only 11.4 per cent had subnormal protein concentrations, that is, less than 6 grams per cent in the blood. When the percentage of patients showing subnormal concentrations of blood proteins was plotted against the hematocrit or hemoglobin concentration, a most important relationship presented itself. (See Fig. 255.) As the deficit

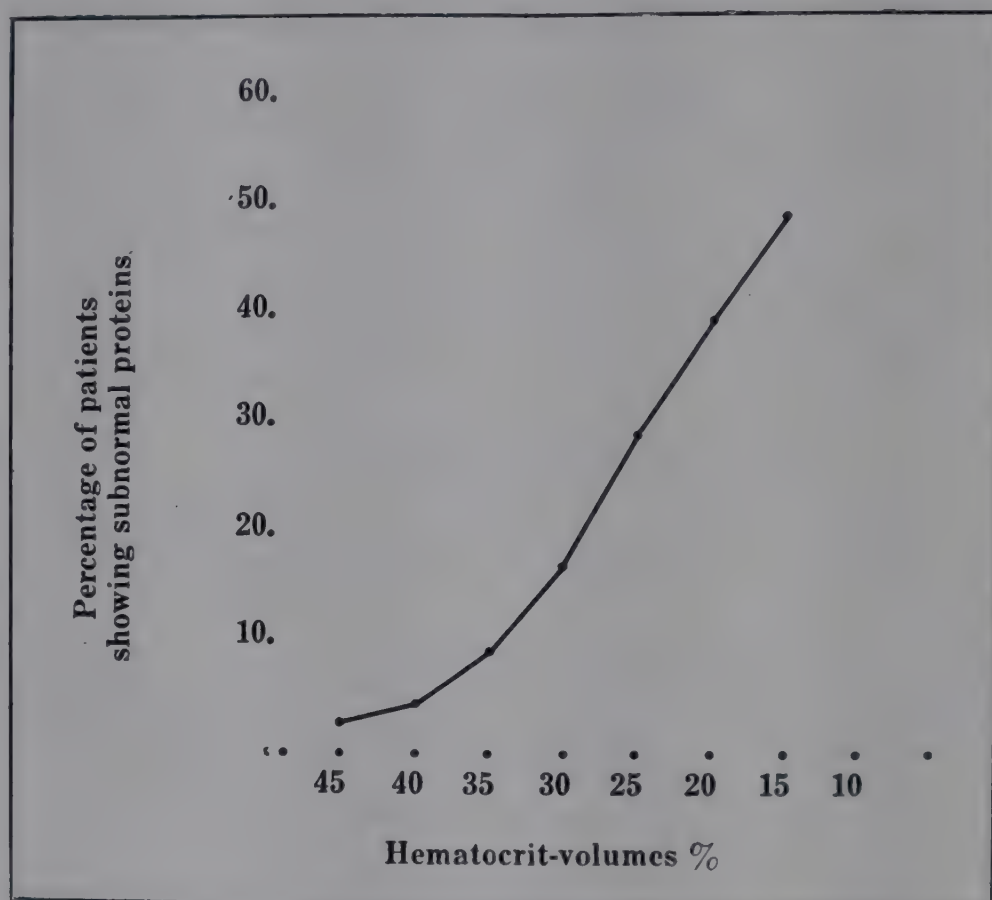


Fig. 255.—(From Agress, Harry: Washington Univ. Med. Alumni Quart., Jan., 1946.)

in hemoglobin became more marked, the percentage of patients showing a diminution in blood proteins rose; at a level of 40 volumes per cent in the hematocrit only 2.3 per cent of the patients had protein levels below 6 grams per cent, but at a level of 30 volumes per cent in the hematocrit, 38.7 per cent of the patients had subnormal protein concentrations. Whipple³ has demonstrated that the body "guards jealously the fabrication of hemoglobin and given a real need for both plasma protein and hemoglobin, the protein flow favors hemoglobin synthesis, which under these circumstances is always produced in more abundance than plasma proteins." Accepting the finding of Whipple, it is self-evident that the correction of the anemia in a great percentage of military patients was imperative, if they were to be converted from the traditionally poor surgical risks to better prospects for surgery. Agress

³Whipple, G. H.: Am. J. M. Sc. 203: 477-489, 1942.

called attention further to the observations of Elman⁴ that many patients have approached protein depletion long before the concentration of total proteins in the blood has become subnormal. He also suggested the importance of ingested protein on the status of body proteins and speaks of depletion of protein resulting from "acute protein starvation." In the injured soldier there is protein starvation inherent in the traditional management of the surgical patient. The gaunt appearance of many of these patients made this fact self-evident. Lyons called attention to this nutritional depletion and stated that weight losses varying from five to thirty kilograms had been observed in wounded soldiers. Agress properly assumed that in the therapeutic approach to these patients correction of anemias as a means of restoring more normal blood relationships is urgent. He treated these patients with as much blood as was necessary to restore the hemoglobin to normal instead of waiting for the slow process of spontaneous hemoglobin synthesis, usually aided and abetted by foodstuffs, iron, or blood in minimal quantities. Hemoglobin is not supplied by plasma. By giving adequate amounts of blood, the hemoglobin hunger of these patients was appeased and great progress was made in restoring normal physiologic relationships. When hemoglobin deficits were quickly restored, the patients were transformed from poor surgical risks to excellent ones. In his hospital experience in the military zones where he served, shock was practically nonexistent after the institution of the program of using large amounts of whole blood.

How much blood is needed? Five hundred cubic centimeters of whole blood were required for every increment of 3 volumes per cent deficit in hematocrit below 45 volumes per cent in order to restore normal hemoglobin values. The progress of the blood status of each patient was followed by serial copper sulphate determinations and adjustments in the amount of blood given made according to the results of these follow-up studies. Almost unheard of quantities of blood were used. For instance, during the twelve-month period preceding the reparative surgery program in his hospital, Agress stated that 213 transfusions were given. For a similar period of time during which the program was in effect, 9,619 transfusions were given, despite the fact that the surgical admissions were only one and one-half times those of the previous period. One patient required as many as sixty transfusions.

These facts are quite interesting and important in civilian as well as in military practice. It is obvious that not enough whole blood is being given to patients preparatory to surgical operation. It is recommended that the copper sulphate method be used as outlined by Agress in civilian surgery preparatory to operation and that adequate amounts of whole blood be utilized. Often too little whole blood is given to patients and equally often too much is unnecessarily given.

In addition to the hemorrhagic states, transfusion of whole blood is necessary in anemias of blood loss, anemias with increased erythrocytic destruction of hereditary or acquired nature. The basic principle of blood transfusion utility in anemia is a replacement therapeutic procedure. The usefulness of transfusions in anemias due to increased destruction or excessive hemolysis of erythrocytes is seen in the good results in familial hemolytic jaundice,

⁴Elman, R.: *Ann. Surg.* 120: 350-361, 1944.

sickle-cell anemia, erythroblastosis, and many acute and chronic types. There are other types of anemia where blood transfusions are welcome; namely, Cooley's erythroblastic anemia.

Transfusions are indicated in leukemia, although no particularly brilliant results are seen in the chronic leukemias except, perhaps, relief of the associated anemia. In regard to leukopenia, the rationale for transfusion is only of a supportive nature. According to Doan,¹ transfusion is proper in neutropenic states because normal blood contains nucleotides which stimulate the formation of these neutrophilic cells. Transfusions have been used in agranulocytosis with varying success in different hands.

In infections, blood transfusions have been used in many acute and chronic conditions. Small multiple transfusions have been used in infections with good results. In intoxications and poisoning, whole blood transfusions have been valuable; plasma injections, however, have been even more useful.

The **contraindications for blood transfusions** are principally acute pulmonary edema, cardiac decompensation, and massive pulmonary embolism or infarction.

Heparin and Transfusion.—The Scandinavians have been using heparinized blood in transfusions for a number of years. It eliminates the disadvantages of sodium citrate as an anticoagulant and greatly simplifies the procedure of transfusion when given directly to the donor. It may be added to the donor's blood in vitro or injected into the donor during the technic of transfusion.

The Direct Method of Blood Transfusion.—There are various methods of making a direct blood transfusion dependent upon the type of apparatus used. The first modern method for blood transfusion using an apparatus was the one described by Unger.² This apparatus permitted the withdrawal of blood from the donor and the simultaneous injection of saline into the patient. A stopcock was then turned and the reverse situation resulted; blood could be injected into the patient and saline simultaneously injected into the donor. It was this immediate flushing with saline of that part of the instrument not used for the aspiration or injection of blood that insured freedom from clotting.

Another method is that using multiple glass syringes. In this method, there is a battery of about twenty 20 c.c. glass syringes. These are sterilized and placed on a sterile table close to the donor. An assistant inserts a needle into the donor's vein and the operator inserts a needle into the recipient's vein. The first 20 c.c. syringe is half filled with warm saline, that is, about 10 c.c., and 10 c.c. of blood are withdrawn. The syringe is quickly disconnected and connected up with the needle in the recipient's vein and the first syringe material is injected. While this is being injected, the assistant takes the next 20 c.c. syringe and fills it up with blood, passes it to the operator who in turn injects it. In this way, the operation is carried out and the operator, by counting the number of syringes alongside his table, sees at all times just how many cubic centimeters of blood he has injected.

¹Doan, Charles A.: J. A. M. A. 99: 194, 1932.

²Unger, L. J.: J. A. M. A. 65: 582; 1915; J. A. M. A. 65: 1029, 1915; J. A. M. A. 69: 2159, 1917.

Indirect Method of Blood Transfusion

The best method of indirect blood transfusion is one which makes use of vacuum bottles, a closed circuit to prevent bacterial contamination, and disposable tubing, needles, and other equipment. Such equipment is manufactured by Baxter Laboratories, Inc., Morton Grove, Ill., and also by Abbott Laboratories, Chicago, Ill., and Cutter Laboratories, Los Angeles, Calif. The Baxter equipment is described here. See Fig. 256.



Fig. 256.—Transfuso Vac containers. Blood collected in these containers may be stored up to 21 days and administered as whole blood, or plasma may be prepared from the stored blood. The remaining cells may be used for red cell infusion. Capacity of containers are, from left to right, 150 c.c., 250 c.c., 1,000 c.c., 500 c.c. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

The use of rubber tubing in blood transfusion is now obsolete. We have therefore eliminated all reference to directions for cleaning and sterilizing such tubing. Since some institutions still use the metal valve instead of a completely disposable unit, directions for using this valve are included in this chapter.

Directions for Obtaining Blood, Using a Metal Valve

Remove the expendable Baxter Transfuso donor set from its sterile wrappings.

Close the sterile valve by turning the knob clockwise. Fig. 257 shows the Transfuso Valve.



Fig. 257.—Baxter Transfuso Valve. (Courtesy American Hospital Supply Corporation, Chicago, Ill.)

Tear the metal seal from the Transfuso-Vac, lift off the metal disk, and remove the top plastic diaphragm aseptically. Fig. 258.

Insert the needle of the Transfuso Valve perpendicularly through the sealing diaphragm and stopper at point marked "X" on the top of the Transfuso-Vac. Immediately after insertion, recheck the valve to make certain it is still closed.

Attach the disposable tubing and needle.

Choose the vein to be used and carefully prepare the overlying skin with iodine. Make compression on the upper arm, preferably with a blood pressure cuff, and insert donor needle into the vein.

Open the valve slightly by turning the knurled knob counter-clockwise.

While the blood is flowing, agitate the Transfuso-Vac to mix the blood and citrate and prevent coagulation.

When the desired amount of blood has been drawn, close the valve. Remove the blood pressure cuff, withdraw the needle from the vein. Remove the valve from the Transfuso-Vac and salvage the blood from the tubing for serologic study by opening the valve and allowing the blood to run into the sterile pilot tubes, one with and one without anticoagulant. Shake the tube containing the anticoagulant.



Fig. 258.



Fig. 259.

Fig. 258.—Closure of Transfuso-Vac and Plasma-Vac containers. Note the metal identification disk under which is a plastic disk which protects sterility, and a thin rubber disk to protect the surface of the stopper. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

Fig. 259.—Baxter blood and plasma container stopper. The puncture position is marked with an "X." This thicker portion of the stopper will reseal after use so that the vacuum is retained after the blood unit is filled. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

Agitate the Transfuso-Vac a minimum of 3 minutes, assuring adequate mixing of the citrate and blood, thus safeguarding against clotting.

Before placing the filled Transfuso-Vac in a refrigerator overnight (as for the preparation of plasma, etc.), break the remaining vacuum with a sterile air filter. This is a needle connected with a piece of rubber tubing to a glass outlet containing cotton, the entire apparatus having been previously sterilized by dry heat.

To Clean the Transfuso Valve.—

Remove the valve stem. Do not remove the valve nut and needle in cleaning. If needle replacement is necessary, be sure to replace the nut tightly. Remove any residual clots which may remain in the valve needle or valve proper by loosening with a stylet from a 15 gauge needle.

Wash thoroughly in a solution of tincture of green soap. If clots remain, immerse in concentrated ammonium hydroxide for 10 minutes and then rinse in triply distilled water. Never use tap water to wash the valve.

Reassemble, using a liberal quantity of U.S.P. glycerin in the grooves of the valve stem and on the threads of the valve barrel.

Leave the Transfuso Valve one-quarter open during autoclaving. If a valve "freezes," return it to the manufacturer for repair.

Rinse with 5 to 10 c.c. of sterile saline to remove any water which, if it remained, would cause hemolysis.

Wrap and sterilize in an autoclave at 15 pounds pressure for 20 minutes.



Fig. 260.—Making venepuncture for collection of blood, using Baxter Transfuso-Vac with sterile expendable Plexitron R20A blood collection set. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

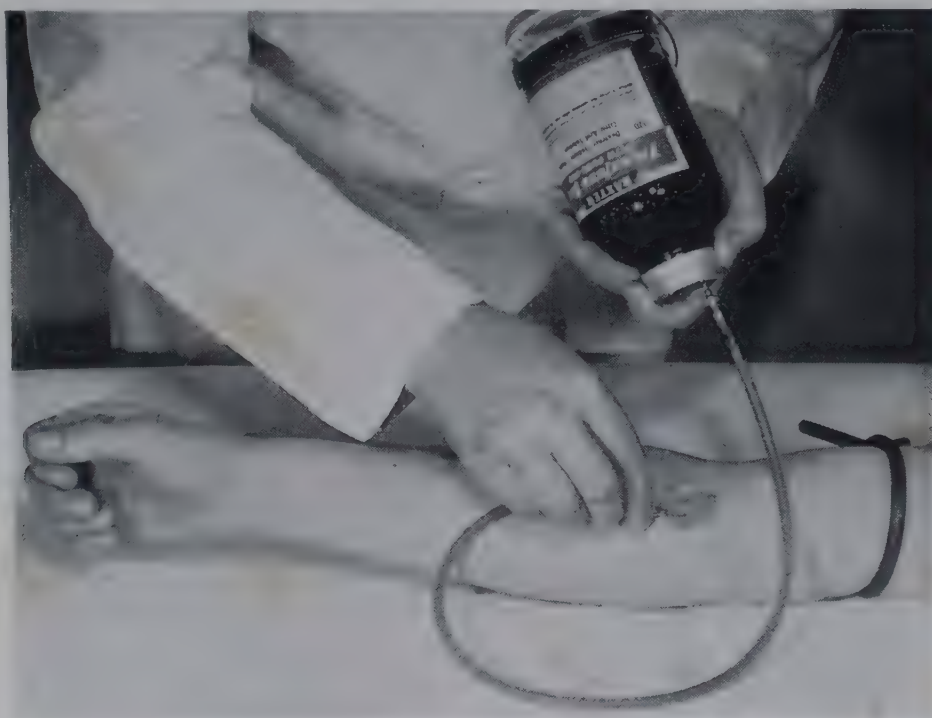


Fig. 261.—Blood collection nears completion. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

TO WITHDRAW BLOOD USING DISPOSABLE UNITS

Prepare the donor as above. To obtain blood, proceed as above, except that no valve is needed if the Transfuso donor set of the expendable type is used. Method of using such sets is shown in Figs. 260 and 261.

To obtain blood for blood grouping and other serologic tests, clamp the Plexitron tubing with the aluminum disk, release tourniquet, withdraw needle from the rubber stopper, insert into vacuum pilot tubes, one of which contains a dry anticoagulant, and remove enough blood from the donor's vein to fill the tubes about three-quarters full. Then clamp the tubing, withdraw the needle from the vein, and cleanse the skin in the usual manner. Shake the tube containing the anticoagulant.

TO ADMINISTER WHOLE BLOOD

Invert the Transfuso-Vac momentarily to moisten the hole in the rubber stopper. With a pair of sharp scissors, cut the rubber diaphragm at the extreme edge, farthest from the air tube.

Tear the diaphragm straight across, exposing both holes in the rubber stopper.

Place the aluminum disk (control clamp) on the Plexitron tubing, Fig. 262, of the administration set, Fig. 263.

Insert the drip device of Plexitron R18 administration set into the bottle of blood, suspend the bottle by its bail, and fill the drip device with blood as shown in Figs. 264 and 265.



Fig. 262.



Fig. 263.

Fig. 262.—Aluminum disk, used as a control clamp for administering blood, plasma, serum, etc. The flow of fluids can be accurately controlled. The disks are used once and then discarded. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

Fig. 263.—Sterile expendable Plexitron R18 set for administering blood, plasma, or serum. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

Establish the blood level in the drip device, then open the clamp to fill the remainder of the set and to remove all air from the tubing.

Attach the vein needle to the Nylon needle adapter and again be sure that there is no air in the needle or the tubing.

Make puncture of the patient's vein in the usual manner after disinfecting the skin, and proceed with the transfusion.

Adjust the clamp to control the rate of blood administration (Fig. 266).

If one desires to start the transfusion with saline or to mix saline with the blood, or to follow the transfusion with an injection of saline, connect the Transfuso-Vac to the bottle of sterile saline, connecting them with a "Y" outfit leading into a filter drip. This "Y" outfit is also a disposable unit. See Figs. 267, 268, 269.

SUGGESTED AND ACCEPTED PROCEDURES

Some such acceptable technic as tincture of iodine and alcohol should be vigorously used in preparing overlying skin. Site for insertion is then anesthetized by an injection of 1 or 2% Novocain intradermally. Place blood pressure cuff on donor's arm, setting the pressure at slightly below diastolic level. Insert needle in donor's vein through anesthetized skin with bevel of needle up.



Fig. 264.—Drip device of Plexitron R18 administration set is filled with enough blood to cover the filter by “milking” the rubber tip. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)



Fig. 265.



Fig. 266.

Fig. 265.—Opening clamp to fill remainder of set before attaching vein needle to Nylon needle adapter. This is done after the blood level in the drip device of Plexitron R18 administration set has been established. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

Fig. 266.—Adjusting clamp to control rate of blood administration. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)



Fig. 267.—Sterile expendable Plexitron R19 "Y"-type set for administering blood, plasma, or serum with solutions, alternately or simultaneously. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)



Fig. 268.



Fig. 269.

Fig. 268.—Expendable "Y"-type administration set, with aluminum disks in position. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

Fig. 269.—Plexitron R19 expendable "Y"-type set for administration of blood, plasma, or serum with solutions, alternately or simultaneously. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)



Fig. 270.



Fig. 271.

Fig. 270.—Fuso-Flo stopper available for Transfuso-Vac containers. Although it does not replace the blood filter, it acts as a preliminary strainer for blood or plasma during administration, and prevents the fibrin of stored blood from retarding the flow. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

Fig. 271.—Baxter Plexitron R48 combination administration set and pressure pump, which enables administration of blood at the normal rate or rapidly under pressure. The rate of administration is determined by pumping action. The R48 *cannot pump air*, since it operates with fluids. It contains a filter that permits rapid administration through mesh fine enough to remove particulate matter. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)



Fig. 272.—Aspirating plasma. The plasma will be drawn off into a vacuum container. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

If blood does not flow immediately, close Transfuso Valve (or clamp if disposable unit is used) and improve position of needle. Improper placement of needle may cause collapse of tubing. It will be expanded again by flowing blood when needle is properly placed in vein. This is a good check on needle position. When blood is flowing freely the Transfuso Valve or clamp is slowly opened further and the flow of blood gradually increases until desired rate is established. Average rate is between 75 and 125 c.c. per minute.

Usual causes of insufficient flow of blood are:

- a. Needle is not in good position in the vein.
- b. Pressure in the blood pressure cuff is too high or too low. Occasionally, a better flow can be maintained if donor slowly opens and clenches hand.

Note: If it becomes necessary to remove needle from vein always close valve before removing needle. After the needle has been removed from the vein, hold it as high as possible above valve and very slowly open the Transfuso Valve allowing blood remaining in tubing to be drawn into Transfuso-Vac. Instantly close valve when blood is entirely out of tubing. Re-insert donor needle and slowly re-open Transfuso Valve, so as not to collapse vein.

Blood From Cadavers for Transfusion

Considerable interest was evoked by the announcement of Judin, chief surgeon of Sklifasovski Emergency Hospital in Moscow, who within 3 years performed more than 300 blood transfusions, taking the blood from dead individuals, storing it, and then injecting it into living patients. Judin's work has been referred to by Gosset, chief surgeon of Salpetriere Hospital in Paris, in a publication entitled "La Transfusion du Sang de Cadavre a l'Homme."

In Moscow whenever an acute accidental death occurred from suicide, homicide, or in an industrial accidental way, the dead subjects were immediately brought to one hospital where their entire venous blood was removed and stored. The cadaver was strapped on his back on the operating table, covered with sheets and sterile drapings, just as would be done in the case of a major operation on the living. The neck was left exposed and prepared with sterile precautions, iodine and alcohol, etc. The jugular vein was exposed and two slits made into the vein, one for a cannula to receive blood from the head, the other from blood below. These cannulas were connected with glass sections and rubber tubing, terminating in sterile glass jars stoppered with cotton. No citrate was added. The blood clotted, was placed in a refrigerator, and within two hours the blood was fluid. The clots had autolyzed. The operating table was tipped forward at a sharp angle and blood permitted to flow by gravity into the jars. The filled jars were kept at refrigerator temperature just one or two degrees above freezing point, and warmed in a water bath at 40° C. just before use.

Judin has kept this blood as long as a month and used it without reaction. Kahn and complement fixation tests are run on the specimens before use.

According to this account of Judin's work, the blood of many cadavers has been tested and found sterile. Of course, the subjects used are persons who have died suddenly, from accidents, suicide, or heart attack. It is claimed that all fresh blood from this type of death is free from bacteria. This raises a question in my mind, for in 1904, I¹ reported an account of a research upon this very question; namely, does the blood of cadavers meet-

¹Gradwohl, R. B. H.: Ann. Inst. Pasteur 18: 766, 1904.

ing death from acute accident or from a sudden illness, contain bacteria? This was in reply to a publication of Simmonds² who claimed that in a bacteriologic examination of the cardiac blood of 1,200 subjects, he found a streptococcus in 95 per cent of all cases, autopsy being performed from twelve to thirty-six hours after death. Canon³ later claimed that Simmonds' work was in error in that cardiac blood alone was examined and that if at the same time venous blood had been examined, it would have been found that venous blood was sterile, even with streptococci in the heart blood. Our own work was undertaken in an attempt to settle this point, and so fifty cases were selected from our service at the Coroner's office in this city, in the main, gunshot wounds of the chest, fractures of the skull, ten cases of valvular leak, three cases of rupture of aortic aneurysm, six cases of nephritis, a few cases of purulent peritonitis from abortions, etc. The time elapsing from death to the moment of autopsy performance varied in our cases from as little as two hours to as long as ten hours. In 78 per cent of these cases, bacteria of various kinds were found in the cardiac blood, in only three cases were bacteria found in blood from the median basilic vein. The bacteria found were streptococci, staphylococci, *Proteus vulgaris*, *Escherichia coli*, pneumococci, pyocyaneus. We concluded that there is a rapid invasion of the cardiac blood soon after death, from neighboring organs, and that bacteriologic results therefore from cardiac blood in the cadaver must be weighed in the balance before being accepted as causative factors in deaths of such cases.

These findings give us pause in accepting this Russian announcement, which, while emphasizing that the blood from the jugular veins is free from bacteria, still does not take into account that blood drainage in Judin's technic, coming from the region of the heart as well as from other parts, makes it possible to introduce these postmortem invaders into the recipient end of the transfusion. Further research should be conducted to determine the possibility of infection in such a technic. It is an interesting communication, however, and deserves study and investigation, for even with our best methods in the modern hospital, sometimes very valuable time is lost in finding a donor for an urgent and almost exsanguinated individual. The modern blood bank should supplant this Russian procedure.

Blood Banks

The so-called "blood bank"^{4, 5} was first established in Russia, but the first in America was at the Cook County Hospital, Chicago, Ill. It was eminently successful and has been adopted in many other places. It is essentially a method of keeping on hand quantities of blood so that the hospital is always prepared to give blood transfusions. Like all banks, it is supposed to be operated on the principle that withdrawal of blood means substitution of other blood, so that the supply on hand is fairly constant. A blood bank is a useful adjunct for an institution, provided it can afford equipment and personnel.

²Simmonds: Virchows Arch. 175: No. 3, 1904.

³Canon: Centralbl. allg. Path. u. path. Anat. 15: 133, 1904.

⁴Fantus, B.: J. A. M. A. 109: 128, 1937; Modern Hospital 50: 57, 1938.

50: 57, 1938.

⁵Fantus, B.: J. A. M. A. 111: 317, 1938.

For the satisfactory operation of a blood bank, equipment and personnel are required. Unless it is under the control of responsible personnel and continuously checked, it is obviously a dangerous addition to any hospital program.

Blood is obtained from the relatives and friends of patients who are transfused. There must be direct contact with the donors. Donors should be between the ages of 21 and 60; in good health; should be free from syphilis as proved by serology and physical examination; chronic alcoholics should be rejected; there should be a history of no recent illness, mild or severe. Donors who meet these requirements must certify in writing that they release the hospital from all liability and they must give permission to use blood as the hospital authorities may elect. The rules governing a donor clinic are as follows:

1. Those in charge must be well trained and well supervised.
2. Simple, uniform technic must be used.
3. Unusual care must be taken in technic to preserve product.
4. Discomfort of donor must be reduced to minimum.
5. Adequate room must be provided and unnecessary delay avoided.

The practice is to draw blood from all donors without preliminary typing or testing. This is deposited in favor of the patient for whom it was given. That means that each transfused patient has an account and he may receive blood until his account has been exhausted. If any patient has more than is needed, this may be transferred to the account of some patient who lacked donors. Withdrawing blood from all donors who volunteer prevents the necessity of taking large amounts from any one person. All samples must be thoroughly tested before being used.

The next step in blood banking is storage of blood in the refrigerator, which is carried out immediately, as undue exposure to room temperature and unnecessary handling cause hemolysis. The optimum storage temperature is 4° C. with a permissible range of temperature from 2 to 6° C. Citrated blood will freeze between 0 and -1° C. Attachment of the refrigerator to an electric circuit separate from that of all other electric appliances is advisable so that current failure will not so quickly occur.

A refrigerator has been devised with a "refrigerator indicator" attached to each flask to indicate whether or not refrigeration has been properly observed during the history of the blood specimen. DeGowin and Hardin⁶ have advised two types of refrigeration indicator and label employing 13 by 100 mm. Pyrex glass tubes and a mixture of oleic and palmitic acids with a melting point of approximately 50° F. or 10° C. In type I the fatty acid mixture is colored with Sudan III. The glass capillary tube is filled with the red liquid at room temperature, chilled to freeze the mixture, and threaded on a card label through which holes have been punched. Melting of the mixture causes it to be lost from the tube. Type II employs uncolored fatty acids separating two glass beads. The melting of the mixture allows approximation of the beads.

According to Kilduffe and DeBakey,⁷ two such indicators were preferred. The first is a mixture of 96 per cent oleic acid and 4 per cent palmitic acid, by

⁶DeGowin, E. L., and Hardin, R. C.: *War Med.* 1: 334, 1941.

⁷Kilduffe, R. A., and DeBakey, A. B.: *The Blood Bank and the Technique and Therapeutics of Transfusions*, p. 234, The C. V. Mosby Co., St. Louis, Mo., 1942.

volume, which has a melting point between 10 and 13° C. This mixture is poured at room temperature into a 13 by 100 mm. serologic test tube to a depth of 1 cm. and a red or blue glass bead is added, which sinks to the bottom. The mixture is then solidified by plunging the tube in an ice bath and a similar glass bead is placed on top. The tube is then tightly corked and attached to the blood flask by a rubber band. A slip of cord or heavy paper showing the source and date of the blood collection can also be inserted into the tube. During storage, if the temperature rises to the melting point of the solidified fatty acids, the glass bead on the surface sinks, and the presence of both beads together at the bottom indicates refrigeration failure, in which case the flask should be discarded. The second type of indicator described by these authors utilizes the same fatty acid mixture colored by the addition of Sudan III. At room temperature the liquid mixture is sucked up into a 3 cm. length of glass tubing of 4 to 5 mm. diameter. The mixture is then frozen solid by immersion in ice water, the tube is threaded through two holes of a label card, and the card is inserted into a previously chilled 13 by 100 mm. test tube, which is then corked and attached to the blood flask. As contact between capillary tube and test tube is prevented by the label card, a layer of air insulation is thus provided. If failure of refrigeration allows the temperature to rise to the melting point of the fatty acid mixture, after ten to twelve minutes the red mixture runs out of the capillary tube.

The blood is checked daily by the banker for evidence of hemolysis. The equipment for procuring blood is essentially the same as that described for blood transfusion. Blood is dispensed daily during the regular banking hours except for emergency transfusions. Request to the laboratory to determine the type of the patient is made and request, with patient's blood, is sent to the bank, where cross-matching is done. All blood in the blood bank should have been previously grouped and subgrouped, including the Rh type.

Blood should be transfused immediately. It should be handled not more than is necessary. An intern should remain with the transfused patient for the first fifteen minutes. All reactions should be reported to the Director of the blood bank service. All reports of transfusions should be returned to the blood bank within twelve hours after transfusion.

It must be repeated here that all blood in the blood bank should be proved to have come from nonsyphilitic and nonmalarial patients. Routine culture tests should be done to detect bacterial contamination.

No blood should be used that has been kept longer than three weeks. Outdated bloods may be used to procure plasma, following the practice of removing plasma from the whole blood as outlined on page 1122. In addition, the red cells may be saved for use in special medical and surgical conditions.

In connection with the question of blood banks, attention is to be called to the following section contributed by the American Red Cross on the American Red Cross Blood Program. This program was instituted in this country to provide blood transfusions for the smaller hospitals which cannot afford to conduct a blood bank of their own. There are now forty-five Regional Centers.

American Red Cross Blood Program*

The American Red Cross had its first experience in blood procurement during World War II when it collected approximately fourteen million pints of blood which were turned over to commercial laboratories for processing for the Armed Forces. The Red Cross served only as a collection agency; all other processes were carried out either by the Army or Navy or by laboratories under contract to them. Following World War II, the Red Cross expected to get out of the field of blood collecting and did. However, technical advances made during the war increased the demand for blood, particularly by physicians who had had both blood and plasma and occasionally serum albumin available. Because of this demand some Red Cross chapters were permitted to operate so-called "walking blood banks" on a community basis; others operated collection centers. Although these permissive programs served a need in limited areas, the need for blood was critical in many others. Upon the request of the Army and Navy, Veterans Administration, American Hospital Association, American Medical Association, U. S. Public Health Service, and other allied organizations, the Red Cross investigated the possibility of a national blood program.

With the endorsement in principle of its program, the Red Cross opened its first Regional Blood Center in Rochester, N. Y., in January, 1948. The St. Louis Center was the twelfth to open. Its opening date was August, 1948. There are now 45 Regional Centers. Fifteen Defense Centers came into being for the collection of plasma after the Korean conflict began.

A Regional Center opened in a particular metropolitan area after receiving approval of the American Hospital Association, Catholic Hospital Association, any hospitals not belonging to either, Medical Society, Public Health Service, and the Red Cross Chapter in that city and those in the surrounding areas expecting to be served by mobile units. Defense Centers opened with the same approvals plus an understanding that blood collected by these centers would not be distributed to hospitals in the area but would either be used as whole blood in Korea or processed by commercial laboratories as plasma, serum albumin, or immune globulin for the Armed Forces. Blood quotas of approximately 5 per cent of the population per year were set for Regional Centers and about half that for Defense Centers.

The original aim of the Red Cross Program was to make blood and blood derivatives available to all whenever and wherever needed. It was realized by those outlining the program that this ambitious aim could be reached only through complete cooperation of the American public, which really is the American Red Cross. Cooperation had to come through many channels; first, people must be donors so that the product would be available; second, hospitals and doctors must be willing to use blood in a highly intelligent and conservative manner; third, technical personnel must be willing to learn enough about blood to be able to administer it with the least amount of hazard. Hospital administrative people, technicians, nurses, Red Cross personnel had to be able to explain the simple operating details of the program to recipients.

*This section has been prepared by the St. Louis Regional Blood Program, American Red Cross, St. Louis, Mo.

These details are primarily concerned with why the administration charge is made and what it covers, why some hospitals continue to operate their own blood banks and receive little Red Cross blood, and why some hospitals receive their total supply from Red Cross, and other such questions.

The technical problems have been well handled by physicians and laboratory technicians. Those not previously accustomed to having blood available have sought and obtained the necessary information for safe administration. In explaining the technical aspects of the program, we must pay tribute to the outstanding experts in American medicine who outlined the technical operation of the program and who continue to give it direction. The official Medical Advisory Committee of the Red Cross Blood Program is the same as the Blood and Blood Derivatives Committee of the National Research Council. This



Fig. 273.—In a Red Cross Blood Center, a nurse replaces her equipment after taking a pint of blood from a donor. A Red Cross volunteer nurse's aide lends assistance.

committee participated in the writing and approved the technical manual used by the various centers. The technical operation of each center has the approval of the National Institutes of Health and has a yearly inspection. The technical aspects of the program may be divided into four units for the sake of discussion: donor requirements and acceptance; collection technique; laboratory work; and distribution.

Donor requirements may be divided into those requirements set up for the protection of the donor and those requirements set up for the protection of the recipient. We will consider first those **requirements protecting the donor**:

Age.—Persons between the ages of 18 and 59 are acceptable; persons from 18 through 20 must have written parental permission unless married and then the written permission may come from the spouse.

Temperature and Pulse.—Temperature and pulse are checked and any abnormalities are reported to the attending physician.

Weight.—Donors must weigh at least 110 pounds.

Blood Pressure.—In general, persons with systolic pressures between 100 and 200 are acceptable. The physician checks on the medical history of some in this group and may reject them on his findings.

Hemoglobin.—12.5 grams per 100 c.c. for women and 13.5 grams for men is considered the lowest level for acceptance. Those falling below this level are rejected by the physician. The hemoglobin test is made by the Phillips and Van Slyke copper sulphate specific gravity technic. A disposable lancet is used in pricking the finger. All lancets and needles used are of the disposable type in order to eliminate the danger of transmitting homologous serum jaundice from one donor to another.

History.—

In order that no one ill of some acute disease or chronic illness be taken as a donor, each donor is asked questions concerned with the following conditions: illness in past month, surgical operations in past six months, tuberculosis, diabetes, rheumatic fever, cardiovascular disease, kidney disease, persistent cough, pain in chest, shortness of breath, fainting spells, and convulsions. Any person giving a positive answer to any of these questions is automatically referred to the attending physician who accepts or rejects him on an individual basis. Extreme apprehension is occasionally the cause for rejection. The physician makes an attempt to give the prospective donor a good understanding of why he is rejected, whether or not he may give blood at another time, and what course to follow.

Briefly, the points for consideration for **protection of the recipient** are:

Blood or Plasma Transfusion.—No donor is accepted who has had a blood or plasma transfusion in the past six months.

Malaria or Antimalarial Therapy.—Donors who have had a history of malaria or who have received intensive suppressive therapy but who have had neither a clinical attack nor suppressive therapy in the preceding two years may be accepted for donations to be used as whole blood. Donors who have had neither a clinical attack of malaria nor intensive suppressive therapy in the past six months may be accepted for donations to be used as plasma or plasma fractions only.

Undulant Fever.—Persons having had undulant fever within the past two years are rejected. If the disease occurred more than two years ago the donor is acceptable.

Jaundice.—Persons having had infectious hepatitis or homologous serum jaundice within the past two years may not be donors. Persons having had contact with either disease in the past six months may not be donors. This blood shall be clearly marked for derivatives only.

The problem of contact with homologous serum jaundice and infectious hepatitis presents a real problem when considering laboratory technicians, nurses, physicians, and other hospital personnel as donors. There have been

many recent reports of "needle jaundice" in persons doing venepunctures and examining the blood of patients. We would like to caution all laboratory personnel that the greatest of care should be exercised to protect themselves from infection by the blood of any patient. Any break in the skin should be carefully protected.

Allergy, Asthma, and Hay Fever.—The transmission of nonspecific antibodies by stored blood does not present the great problem that it does in fresh blood; nevertheless a person who feels well but gives a history of recent allergic manifestations is accepted for plasma or derivatives only.

Immunizations.—In general, a person may give blood two weeks after receiving immunizations. If a shorter interval intervenes, the blood will be marked for plasma.



Fig. 274.—Technician in the laboratory of the St. Louis Regional Blood Center making blood grouping tests, including the Rh factor. This is a mass operation. There were 410 blood specimens on the day the photograph was taken; each two represent a blood donor. In the background can be seen tubes lined up for the confirmatory tests using serum against known A and B cells.

The actual collection technic depends to a degree on the type of equipment used. At the present time, all venepuncture sets and bottles used are of the disposable type made by commercial houses. Whole blood collections are taken in bottles containing 110 c.c. of acid-citrate-dextrose (ACD) solution. Blood for plasma is taken in bottles containing 50 c.c. of 4 per cent sodium citrate. The rubber stopper is prepared with 2 per cent aqueous iodine and 70 per cent alcohol before insertion of needle. The arm preparation is done with 50 per cent aqueous green soap, 2 per cent aqueous iodine removed with 70 per cent alcohol. Intracutaneous 1 per cent procaine is injected before the venepuncture is performed if the donor requests it. Bleedings for ACD and citrate are done in 5 to 7 minutes. Blood collected in ACD is care-

fully agitated approximately five times during withdrawal and is thoroughly shaken after withdrawal is completed. Blood taken in citrate is not agitated unless separation of blood from anticoagulant is noted. Immediate refrigeration is instituted in both cases.

We shall outline the procedures as carried out by the St. Louis Regional Blood Program. Blood collected in ACD solution is distributed only after the following laboratory work has been done:

1. Determinations of A-B-O groups by known sera and known cells.
2. Determinations of the Rh factor, **Rh₀** (D), **rh'** (C), and when sera are available, **rh''** (E).
3. Serologic test for syphilis is the VDRL.
4. Titrations have been discontinued on all group O donors. Group O, ACD bottles are labeled "Isoagglutinin Titer Not Determined." We discourage the use of group O blood as universal donor blood, but realize that in cases of extreme emergency it must be used. Titrations should be carried out in hospital laboratories if group O blood is to be used as universal donor.

A-B-O grouping and Rh tube typing tests are made on blood taken in sodium citrate solution. All other laboratory work is performed in the commercial laboratory.

The A-B-O groupings and Rh typings are done by the slide method from clotted samples collected at the time of venepuncture. A Clay-Adams viewer is used for the Rh tests. Commercial sera of a potency meeting National Institutes of Health standards are used.

Although the standard three-tube Kahn is used for the serologic test for syphilis in some Red Cross Blood Centers, many others are converting to the VDRL syphilis test. Donors with positive tests are followed up as recommended by the State Health Department.

The laboratory procedures are of the simplest type commensurate with the greatest accuracy possible. Perhaps the greatest problem for new personnel and a continuously troublesome problem for the experienced technician in mass typing is that of keeping all determinations related to one unit of blood carefully identified. The elimination of the human error in mixing samples and numbers relating to samples must be accomplished. In American Red Cross Blood Centers, we have found the use of one number to identify a given sample and the use of one laboratory worksheet with no copying to be most helpful in preventing the most common errors in transcribing. "Reverse grouping" using unknown sera plus known A and B cells (2 per cent) is made as a check against the original grouping tests. The value of two technicians reading the same test cannot be overemphasized.

Labeling, storage, and distribution of blood is the final unit for consideration. Every bottle is properly identified as to contents, including the formula of the anticoagulant, date of expiration (21 days from collection for ACD), name and address of collecting agency, results of laboratory tests, i.e., serology, grouping, and typing. A whole blood number corresponding to that on the donor's medical history card appears on the bottle label and can be used to trace any unit of blood to the donor. Each ACD bottle has a pilot tube of

clotted blood taken at time of venepuncture securely taped to it. This tube is taped to the bottle prior to the venepuncture and is not removed at any time in the Center.

Blood is stored in a temperature of 4° to 6° C. in a refrigerator used for this purpose only and which has a constant recording device and alarm system.

The blood is distributed by the American Red Cross in precooled iced cases.

Untoward Reactions Following Blood Transfusions

Up to the time of the appearance of the publications of Landsteiner,¹ there had been repeated grave manifestations of agglutination and hemolysis from the mixture of incompatible bloods. His work, showing the blood groups of human beings, laid the foundation upon which has been built the present relatively safe and secure method of blood transfusion as it is practiced today. Reactions sometimes occur due to errors in blood grouping and the consequent transfusing of blood of an incompatible group. Wiener et al.* discussed their experience with reactions in more than 3,000 transfusions. They divide transfusion reactions into two main groups; first, those due to some specific incompatibility between the blood of the patient and that of the donor, and, second, those due to nonspecific agents, the latter constituting the vast majority.

Attention has frequently been called to the danger of using group O indiscriminately as a universal donor because of an occasional hemolytic reaction. Levine and Mabee pointed out that in group O donors with high isoagglutinin titers the dilution of donor's serum in the patient's circulation might not be sufficient to prevent hemolytic reaction; in fact, cases have been reported of hemolytic reactions following use of such dangerous universal donors. It is reasonable to believe that such instances of reactions are certainly high when universal donors are used routinely. It is known that group O is characterized by its incompatibility with A and B.

The work of Wiener and Peters and Levine has shown that the Rh agglutininogen with its consequent anti-Rh agglutinin development is definitely a factor which causes many reactions in blood transfusions. This has been fully explained on pages 1024 ff. In other words, the Rh intragroup isoimmunization must be taken into account to explain the reactions that occur in pregnancy from using an Rh-positive donor for an Rh-negative patient. This also explains the reactions in repeated transfusions where Rh-positive blood is injected into Rh-negative individuals.

The so-called "hemolytic" reaction may occur after the administration of a small amount of blood but usually occurs after introduction of 50 c.c. or more of blood. Such patients complain of tingling sensation or a sense of great discomfort and anxiety, fullness in the head, precordial oppression or a constricting feeling in the chest and difficulty in getting their breath, and severe pain in the back of the neck and the chest and especially in the lumbar region. Signs of collapse follow with rapid feeble pulse, cold clammy skin, marked flushing of the

*Wiener, A. S., Oremland, B. H., Hyman, M. A., and Samwick, A. A.: 11: 102, 1941.

¹Landsteiner, K.: Zentralbl. f. Bakt. 28: 357, 1900; Wien. Klin. Wchnschr. 14: 1132, 1901.

face, dyspnea, cyanosis, drop in blood pressure, dilatation of the pupils, and nausea and vomiting. Hemoglobinuria frequently develops with anuria and jaundice later.

It has been said that the commonest cause of fatality in blood transfusion is introduction of incompatible blood. Death in these cases may be due to multiple thromboses, including the capillaries, but sometimes death occurs due to exsanguination because of the hemorrhagic tendency following a hemolytic reaction. It is, however, manifest that the most common fatal mechanism after the injection of incompatible blood is the comparatively slow and undramatic onset of renal insufficiency. This is accompanied by anuria and uremia and is always of the gravest importance. The mechanism involved in the suppression of renal function after hemolytic transfusion reactions is not settled. Experimental work by many writers has shown that precipitated hemoglobin does block the tubules in these cases, but, in some cases of fatal transfusion anurias, there is no such tubular obstruction. This matter has been well discussed by Peters,² who reported a case of anuria following hemolytic reaction to blood transfusion, with recovery following splanchnic block. He discussed the various theories of pathologic changes in the kidneys and noted that the kidneys of patients dying of transfusion anurias often present points of similarity to the kidneys observed in the so-called "reflex anurias," in which latter vasoconstriction plays such an important part. It is to be noted that not only has renal vasoconstriction been experimentally produced by intravenous hemoglobin injection, but also in the comparatively few reported autopsies after transfusion anuria, the kidneys are grossly congested and swollen. Wiener³ declares the kidneys are usually found to be swollen, with the pyramids appearing dark in color; thus their vascular status might well be analogous to that of the reflex kidney. Experimentally, irritation of the splanchnic nerves has been shown to cause oliguria and conversely section or anesthetic block will cause prolonged polyuria. Attempts as early as 1922 were made to combat therapeutically the renal vasoconstriction in reflex anuria. Neuwirt⁴ first successfully relieved such anuria by means of splanchnic block. Others repeated this observation. Some have advocated spinal anesthesia. Splanchnic block entails the application of a large quantity of anesthetic solution directly to the splanchnic plexus, which may more thoroughly interrupt the sympathetic flow. The duration of splanchnic block would seem longer than one would expect in spinal anesthesia. In transfusion anurias, splanchnic block is advisable but, as stated by Peters, it is "the key" which unlocks the door to further judicious therapy because the establishment of diuresis, while an essential step, far from insures recovery for the patient. Careful observation of the chemical balance of the patient may often clearly guide the treatment. The kidneys must have a continuously positive water balance. Hence, dehydration must be watched for, prevented, or treated. Saline is best to overcome dehydration for the sodium ion is necessary to fix the water to the tissues. However, because saline is taken up by the tissues, it may not be as good in certain stages of an oliguria as glucose solutions, for the water

²Peters, H. Raymond: *Ann. Int. Med.* 16: 547, 1942.

³Wiener, A. S.: *Blood Groups and Blood Transfusion*, ed. 3, Springfield, Ill., 1943. Charles C Thomas.

⁴Neuwirt, K.: *Ztschr. f. urol. Chir.* 2: 75, 1922.

of a glucose solution is more available for renal excretion. While it is not possible for all laboratories to have equipment to perform routine sodium determination, the sodium level can be roughly surmised from a comparison of the blood chloride level and the carbon dioxide combining power. If the carbon dioxide combining power is high and the blood chloride level is low, then the sodium level is probably near normal. There should be definite attempts made to guard against alkalosis. It must be remembered that, no matter how successfully splanchnic block may relieve renal vasoconstriction, in any given case one may still have kidneys in which widespread tubular epithelial degeneration has developed and the prognosis must be judged accordingly. In Peters' case, there was a hemolytic reaction with prolonged anuria following transfusion of blood of the homologous group. After failure of other measures for nine days, diuresis followed within a few hours after a splanchnic block, and there was ultimate complete recovery of the patient. It is obvious that this case offers evidence in favor of a vasoconstrictive renal element in transfusion anuria similar to that of the so-called "reflex anuria."

There has been a tendency among many writers to believe that in all cases of anuria following hemolytic reaction to blood transfusion, the kidneys present blocked vessels at autopsy. This is not necessarily true. It is probable that more causes are due to a mechanism similar to the reflex anuria than are due to actual mechanical blocking of small renal vessels.

In regard to nonspecific reactions, these are very severe but are rarely fatal. They have the following characteristics: thirty to sixty minutes after the injection, the patient has a severe shock which is followed by rise in temperature. Other patients may complain of chill, and the rise in temperature may be only slight. It was first thought that this was due to the use of citrate but experiences have shown that this occurs with saline solution as well as with citrate solution. Wiener's experience is that one per cent of transfusions is followed by symptoms of an allergic nature, usually a generalized urticaria, with eruptions at times associated with edema. This may be due first to the use of the blood from a donor who may have eaten some food to which the patient is sensitive. It may be due to the fact that some allergic reactions are based on individual differences in the serum proteins. Wiener believes, without full proof, that this may be due to factors such as isohemolysins as, for example, in repeated transfusions of blood even of a homologous group. Rapid injection of blood into the circulation causes a sudden increase in blood volume, which causes an additional load on the cardiovascular system. It causes a distention of the peripheral veins, coughing, fullness in the head, and vertigo. If these symptoms are not heeded and the transfusion is continued, the patient may go into collapse.

A problem which arises in blood transfusion work is how to distinguish the nonspecific chills following a transfusion from those chills which are based specifically on the incompatibility of the donor's blood. The first step is to repeat the grouping and cross-matching tests on samples of blood taken before the transfusion and also on fresh samples of blood to determine whether there has been an error in grouping. A sample taken from the patient after the transfusion should also be examined for hemoglobinemia and for icterus index, and, at the same time, the urine should be examined. Tests for M and N should be

made. If the M and N types of the patient and donor are different, tests on patient's blood with anti-M and anti-N sera will determine whether the donor's blood is still present in the circulation of the patient. If the bloods were incompatible, rapid elimination of donor's cells will have occurred. This test cannot be done if the M and N types are the same in both donor and recipient.

Differential Agglutination.⁵—

The principle of the differential agglutination test is to take a sample of the patient's blood after a transfusion with universal donor blood and test it with serum capable of agglutinating the patient's cells. Since the donor belongs to group O, his cells will not be agglutinated by the specific antiserum. For example, if an individual of group A is given group O blood, a count may be made of those cells not agglutinable by anti-A serum after the transfusion. Group O cells are not agglutinable by anti-A serum, but the patient's group A cells are, and thus it may be assumed that any inagglutinable cells found are the transfused cells. However, it must be remembered that in normal blood, approximately 50,000 cells per cu. mm. are not agglutinable by specific antisera, and this must be taken into account when the test is performed.

The method of tracing the fate of transfused red blood cells was first used by Todd and White⁶ in cattle. Ashby⁷ used the method for the first time in man for patients not belonging to group O and transfused with blood from universal donors.

When the patient receives blood of his own group, the differential agglutination test may be made only provided the M and N qualities of the donor and recipient differ. The results with anti-M serum are more striking than when group O blood is used for patients of groups A, B, and AB, for, if tests are made using anti-M serum on blood containing the M agglutinin, very few cells remain unagglutinated as contrasted with the 50,000 per cu. mm. not agglutinable by anti-A or anti-B sera. For example, if OMN blood is transfused to an ON patient, when tests are made using anti-M serum on the patient's blood after the transfusion, only the donor's MN cells are agglutinated; any unagglutinated cells are the patient's. If the donor is ON and the patient is OMN, the unagglutinated cells in tests with anti-M serum are the donor's.

Landsteiner, Levine, and Janes,⁸ Wiener,⁹ and Dekkers¹⁰ established that the blood cells of the donor survive in the patient's circulation for periods up to 3 to 4 months in the usual case where the patient is transfused with fresh compatible blood either from a universal donor or from a donor of the homologous group. Wiener and Schaefer¹¹ showed that the survival time of erythrocytes in bank blood is roughly in inverse proportion to the period of storage.

If incompatible blood is transfused, it remains in the circulation usually for only a short time,^{12, 13} so that even if the transfusion is tolerated, the trans-

⁵Wiener, A. S.: *Am. J. Clin. Path.* **12**: 189, 1942.

⁶Todd, C., and White, R. G.: *Proc. Royal Soc., Biol.* **84**:255, 1911.

⁷Ashby, W.: *J. Exper. Med.* **29**: 267, 1919.

⁸Landsteiner, K., Levine, P., and Janes, M. L.: *Proc. Soc. Exper. Biol. & Med.* **25**: 672, 1928.

⁹Wiener, A. S.: *J. A. M. A.* **102**: 179, 1934.

¹⁰Dekkers, H. J. N.: *Acta Med. Scandinav.* **99**: 511, 1939.

¹¹Wiener, A. S., and Schaefer, G.: *M. Clin. North America* **24**: 705, 1940.

¹²Burnham: *Arch. Int. Med.* **46**: 502, 1930.

¹³Grove and Crum: *J. Lab. & Clin. Med.* **16**: 259, 1930.

fusion at best is of only transient benefit because of the rapid destruction of blood cells. However, Jervell¹⁴ found incompatible cells in an infant for more than six weeks after a transfusion, and Wiener found group AB cells present in a group O infant 50 days after a transfusion.

The method of making the test is to dilute the patient's blood, before the transfusion, with potent specific antiserum, in the usual manner for making a red blood count. Count the number of inagglutinable cells per cu. mm. in the patient's blood.

Repeat this test after the transfusion.

To determine the number of donor cells per cu. mm., subtract the number of inagglutinable cells before the transfusion from the number after the transfusion, and record the number per cu. mm.

If the count after the transfusion exceeds the count before the transfusion, it is assumed that the transfused erythrocytes are still present in the patient's blood.

Wiener and Peters¹⁵ applied this method of differential agglutination to the study of post-transfusion hemolytic reactions. They found that even when patient and donor are of the same blood group, if the bloods are incompatible due to factors other than **A** or **B**, the donor's cells are eliminated rapidly from the patient's circulation, within a period of a few hours or days, rather than in three or four months. The diagnosis of hemolytic transfusion reactions using this method rests on the assumption that donor's blood has been eliminated from the patient's circulation.

PREVENTION OF HEMOLYTIC REACTIONS

No matter how carefully one groups bloods and performs all the tests for compatibility, certain reactions seem bound to occur. This is possibly due to the fact that there are as yet certain facts about the problem of intragroup reactions which have not been solved. Accordingly, Wiener and associates¹⁶ suggested the use of a **biologic test** to supplement these in vitro tests commonly used. The use of a biologic test was suggested first by Oehlecker,¹⁷ who advised the injection of 10 c.c. of blood, waiting ten minutes, and then proceeding with the remainder of the blood if no reaction occurs. This method did not work out so well because the reaction is often delayed beyond the ten-minute period of Oehlecker. Wiener¹⁶ therefore recommended the following procedure:

Allow 50 c.c. of the donor's citrated blood to run in. After two hours have elapsed, if no reaction has occurred, administer the balance of 100 c.c. of blood. If a reaction occurs, withdraw 10 c.c. of blood from the patient and treat it in the same manner as the pretransfusion citrated sample used for the cross-matching test. Centrifuge the citrated blood samples and compare the color of the plasma in the two tubes. The syringe and needles **must be dry**.

If there is no change in the appearance of the patient's plasma, proceed with the transfusion of larger amounts of donor's blood. If the supernatant plasma of the post-transfusion specimen is distinctly redder than that of the pretransfusion sample, give no more of this donor's blood.

¹⁴Jervell: *Acta path. et microbiol. scandinav.* 1: 201, 1924.

¹⁵Wiener, A. S., and Peters, H. R.: *Ann. Int. Med.* 13: 2306, 1940.

¹⁶Wiener, A. S., Silverman, J., and Aronson, W.: *Am. J. Clin. Path.* 12: 241, 1942.

¹⁷Oehlecker, F.: *Die Bluttransfusion*, Vienna, 1933, Urban & Schwarzenberg.

After 100 c.c. of the donor's blood have been injected, it is possible to demonstrate the survival of the donor's cells by the method of differential agglutination (see page 1112) where the biologic test is negative, in patients having a red cell count of two and one-half million cells or less.

In positive reactions, two hours after the test injection of 50 c.c. of blood, patient may have a severe chill and rise in temperature, but clinical symptoms may be mild or absent. Place more reliance on the appearance of the patient's plasma which will show a distinct rise in icteric index as compared to the pretransfusion sample.

BLOOD PLASMA AS A SUBSTITUTE FOR WHOLE BLOOD IN TRANSFUSION

An enormous amount of literature has been accumulated regarding the use of blood plasma as a substitute for whole blood for transfusion in certain indicated cases. The advantages of plasma are that it can be used without typing, that it may be stored in liquid, frozen, or dried states for months or years and then administered safely without significant deterioration of its component qualities. It is readily available for immediate use and when injected does not increase the concentration of red blood cells in cases of shock and burns where hemoconcentration is already present. Manifestly, the use of whole blood where hemoconcentration is already present is contraindicated.

Historical Data Upon the Use of Liquid Plasma*

Blood plasma as a substitute for whole blood was suggested as early as March, 1918, by Gordon R. Ward¹ in England. He pointed out that one of the chief troubles with whole blood was that the recipient's plasma might hemolyze the corpuscles of the donor. He made the observation that death from hemorrhage was not due to lack of hemoglobin but from loss of fluid, and thus the great need in these patients is for the replacement of the depleted fluid. He suggested that this could be done by the administration of citrated plasma which could be preserved easily and injected safely.

Rous and Wilson² in 1918, while working on hemorrhage in animals, used plasma to replace the blood loss. As a result of their experiments they pointed out that the replacement of red blood cells is not essential because even in severe hemorrhage a sufficient number of cells remains to support life. The loss of blood volume is the important factor. Following gross hemorrhage they were able to restore the blood pressure to normal and maintain it by replacing the removed blood with an equal quantity of plasma. Where hemorrhage is too great, loss of red blood cells is important also.

Mann,³ in 1918, discussed the use of serum for treating surgical shock produced experimentally in dogs. He noted that the parenteral injection of homologous serum produced results which were as good as or even better than any of the methods employed in the treatment of experimental shock. It is

*Many of these historical facts were found in the publication by Lt. Comdr. L. R. Newhouser, U. S. Navy, and Captain D. B. Kendrick, Jr., U. S. Army, Medical Corps, entitled, "Human Plasma" prepared April, 1941.

¹Ward, Gordon R.: *Brit. M. J.* 1: 301, 1918.

²Rous, P., and Wilson, G. W.: *J. A. M. A.* 70: 219, 1918.

³Mann, F. C.: *J. A. M. A.* 71: 1184, 1918.

worthy of mention that his best results were obtained with relatively large doses of serum. He asserted that homologous serum might be of value under conditions where serum could be kept and whole blood could not be obtained.

Strumia⁴ began using human serum intravenously in 1927 for the treatment of severe infections. Later in 1927 he began using plasma because of its simplicity of preparation and its greater yield. It was noted then that even heterologous plasma given intravenously caused no reactions whereas homologous sera commonly caused severe reactions. Gradwohl^{4a} recommended plasma in the treatment of a severe burn case. This difference in behavior of plasma and serum had previously been observed and commented on by Brodie⁵ in 1900. Untoward reactions from serum were thought by him to be due to the process of fibrin precipitation when serum is separated from the clotting blood. This hypothesis continues to be conjectural.

Weech, Goettsch, and Reeves⁶ in 1933 while doing plasmapheresis experiments in dogs were able to produce a normal state in their animals by administering transfusions of normal dog serum.

The use of plasma intravenously in man as a hemostatic agent was reported by Filatov and Kartasevskij⁷ in 1935. The same year Heinatz and Sokolow⁸ used plasma in the treatment of hemolytic shock.

Elliott⁹ in 1936 suggested the use of untyped serum and plasma for the treatment of surgical, obstetrical, or traumatic shock where transfusions were indicated. He rationalized that the need for replacing the lost blood volume was more important than the red blood cells, as the maintenance of osmotic pressure is a function of the plasma proteins. Elliott also propounded the idea that liquid plasma could be stored for long periods without deterioration. The findings of other investigators have supported his contentions.

Fantus¹⁰ in 1937, advocated the use of normal human serum because of its therapeutic and natural immunizing properties. He states that in shock without hemorrhage and in burns the intravenous injection of blood serum is much more rational than that of blood, because these patients usually have an excess of red blood cells per cubic millimeter.

Alovski and Burceva¹¹ published a favorable report in 1937 on the use of plasma as a substitute for whole blood in gynecologic conditions. Mahoney,¹² in 1938, recorded encouraging results with preserved plasma in the treatment of experimental and clinical shock. Elkinton¹³ and McClure,¹⁴ 1939, working separately, obtained good results with plasma in burn cases. Tatum, Elliott, and Nessel,¹⁵ 1939, suggested a technic for the preparation

⁴Strumia, M. M., Wagner, J. A., and Monaghan, J. F.: *Ann. Surg.* **111**: 623, 1940.

^{4a}Gradwohl, R. B. H. Quoted by Hewitt, Walter R.: *J. Missouri M. A.* **38**: 191, 1941.

⁵Brodie, T. G.: *J. Physiol.* **26**: 48, 1900.

⁶Weech, A. A., Goettsch, E., and Reeves, E. B.: *J. Clin. Invest.* **12**: 217, 1933.

⁷Filatov, A., and Kartasevskij, N. G.: *Zentralbl. f. Chir.* **62**: 441, 1935.

⁸Heinatz, S. W., and Sokolow, N. I.: *Zentralbl. f. Chir.* **62**: 1753, 1935.

⁹Elliott, J.: *South. M. & S.* **98**: 643, 1936.

¹⁰Fantus, B.: *The 1937 Year Book of General Therapeutics*, Chicago, 1938. The Year Book Publishers, p. 163.

¹¹Alovski, A., and Burceva, E.: *Monatschr. f. Geburtsh. u. Gynäk.* **105**: 38, 1937.

¹²Mahoney, E. B.: *Ann. Surg.* **108**: 178, 1938.

¹³Elkinton, J. R.: *Bull. Ayer Clin. Lab., Penn. Hosp.* **3**: 279, 1939.

¹⁴McClure, R. D.: *J. A. M. A.* **113**: 1808, 1939.

¹⁵Tatum, W. L., Elliott, J., and Nessel, N.: *Mil. Surgeon* **85**: 481, 1939.

of whole blood substitutes in war conditions. Plasma was recommended as "an ideal substitute for whole blood in the emergency treatment of shock and hemorrhage from war wounds." They outlined the technic of collecting blood in a vacuum bottle, centrifuging the blood, and then aspirating the supernatant plasma into a second vacuum bottle for storage. In 1940 Elliott, Busby, and Tatum¹⁶ recommended the use of dilute, preserved, liquid plasma. The same year Strumia⁴ advocated citrated blood plasma without cross-matching for the treatment of burns and shock. Thalhimer¹⁷ reported favorably on the use of convalescent serum for administering antibodies. Best and

TABLE 120.—THEORIES OF SHOCK*

1. Nervous
Vasomotor exhaustion
Mitchell, Morehouse, and Keen (1864)
Fischer (1870)
Exhaustion
Crile (1897-1920)
O'Shaughnessy and Slome (1935)
Inhibition
Meltzer (1908)
2. Fat embolism
Bissell (1917)
W. T. Porter (1917)
3. Arterial vasoconstriction and capillary congestion
Mapother (1879)
Malcolm (1893-1909)
Starling (1918)
Erlanger, Gesell, and Gasser (1919)
4. Acapnia
Henderson (1908)
5. Acidosis
Cannon (1919)
6. Hyperactivity of adrenal medulla
Bainbridge and Trevan (1917)
Freeman (1933)
7. Exhaustion of adrenal medulla
Sweet (1918)
8. Adrenal cortical insufficiency
Swingle, Piffner, et al. (1933)
9. Traumatic toxemia
Cannon, Bayliss, and British Medical Research Committee (1918)
10. Traumatic metabolites giving capillary atony and tissue anoxia
Moon (1932-1938)
11. Local fluid loss
Phemister (1927-1930)
Blalock (1930)
12. Progressive oligemic anoxia
Harkins (1940)

*Harkins, Henry N.: *Surgery* 9: 231, 447, 607, 1941.

Solandt,¹⁸ discussing their work on experimental shock, reported encouraging evidence in favor of using plasma and serum in preventing shock. Levinson, Neuwelt, and Necheles¹⁹ have shown the value of serum in the treatment of posthemorrhagic shock in experimental animals. Kendrick,²⁰ 1941, advocated the use for military purposes of concentrated and normal dilutions of plasma for the prevention and treatment of shock in the combat zone. Kekwick and

¹⁶Elliott, J., Busby, G. F., and Tatum, W. L.: *J. A. M. A.* 115: 1006, 1940.

¹⁷Thalhimer, W.: *M. Clin. North America* 23: 613, 1939.

¹⁸Best, C. H., and Solandt, D. Y.: *Brit. M. J.* 2: 116, 1940.

¹⁹Levinson, S. O., Neuwelt, F., and Necheles, H.: *J. A. M. A.* 114: 455, 1940.

²⁰Kendrick, D. B., Jr.: *Mil. Surgeon* 88: 97, 1941.

Whitby,²¹ 1941, after using it for the treatment of shock and hemorrhage in air raid casualties in England, concluded that plasma and blood are equally effective in restoring blood volume in injuries of this type.

What Is Shock?—

An understanding of the physiology and mechanism of traumatic shock is necessary to comprehend the advantages of the use of blood plasma over whole blood. What is shock? Many theories have been advanced about shock. They are embodied under the twelve different theories in Table 120.

Three ways of classifying shock have been proposed. The first is determined by origin; i.e., traumatic, operative, burn, obstetric, medical shock, etc. The second classification, according to Harkins,* is faulty in the first instance in that the condition is primary but it is not shock; in the second instance, it is shock but it is not primary. Harkins recommends abandonment of the term primary shock. The third classification is that of Blalock, who divides shock into (1) the hematogenic type, usually called secondary, traumatic, and operative or surgical shock; (2) the neurogenic type, usually called primary shock, with a fall in blood pressure, of which shock due to spinal anesthesia or fainting is a type; (3) the vasogenic type, of which histamine shock is a type, the action being directly on the blood vessels; and (4) the cardiogenic type, in which the veins are distended. This condition is very rare.

The following definitions quoted from Harkins* indicate the multiplicity of opinions on what shock really is.

DEFINITIONS OF SHOCK

Travers (1826)	Shock is a species of functional concussion by which the influence of the brain over the organ of circulation is deranged or suspended.
Delcasse (1834)	An arrest of innervation without which all organs pass into insensibility. (An old way of expressing Meltzer's inhibition theory.)
Gross (1872)	A manifestation of a rude unhinging of the machinery of life.
Piéchaud (1880)	Le choc est un état général plus ou moins grave consécutif aux traumatismes, spécialement aux plaies par armes à feu et aux grands écrasements, caractérisé par l'affaiblissement des pulsations du coeur, l'abaissement de la tension, la pâleur des tissus, un certain degré d'anesthésie joint à la faiblesse musculaire, avec conservation de l'intelligence.
Mansell-Moullin (1882)	A sudden check to the circulation brought about through the agency of the nervous system.
Roger (1892)	Le choc est un état morbide qui peut se produire à la suite de fortes excitations du système nerveux et qui est caractérisé par un ensemble d'actes inhibitoires dont un seul, l'arrêt des échanges entre le sang et les tissus semble constant et indispensable.
Lockhart Mummery (1905)	Surgical shock is a condition produced by exhaustion of the vasomotor centers and the resulting great fall in blood pressure.
Lockhart Mummery (1910)	A condition of lowered blood pressure resulting from exhaustion of the vasomotor centers.
Gray and Parsons (1912)	The reaction of the central nervous system to exaggerated or abnormal afferent impulses.
Morison and Hooker (1915)	A low arterial blood pressure of such duration that recovery is impossible.

*Harkins, Henry N.: *Surgery* 9: 231, 447, 607, 1941.

²¹Kekwick, A., Marriott, H. L., Maycock, W. L. A., and Whitby, L. E. H.: *Lancet* 1: 99, 1941.

DEFINITIONS OF SHOCK (CONT'D)

Brown (1917)	Exhaustion of the food material—the Nissl granules—stored in the nerve cells, more particularly those of the vasomotor centers.
Quénu (1918)	Toxémie traumatique depressive.
Cannon (1919, 1923)	Exemia.
Meek (1926)	A decrease in the effective circulatory volume.
Schuberth (1936)	A peripheral circulatory insufficiency.
Davis (1937)	An anoxemia which is the resultant of such factors as oligemia, lowered blood pressure, diminished flow of blood, and peripheral vasoconstriction.
Tomb (1937)	The collapse of the circulation from overstimulation of the sympathetic nervous system.
Griswold (1938)	A deficiency in circulating blood volume.
Moon (1938)	A circulatory deficiency, neither cardiac nor vasomotor in origin, characterized by decreased blood volume, decreased cardiac output (reduced volume flow), and by increased concentration of the blood.
Devine (1939)	A state of depression of all the reflex arcs accompanied by circulatory depression.
Minot and Blalock (1940)	Peripheral circulatory failure resulting from a discrepancy in the size of the vascular bed and the volume of intravascular fluid.
Selye, Dosne, Bassett, and Whittaker (1940)	A condition of suddenly developing general damage.
Freeman (1940)	The clinical condition characterized by progressive loss of circulating blood volume, brought about by the tissue anoxia which results from inadequate circulation.

The conclusion of Harkins* as to the definition of shock, from the various opinions given in the literature, is that it is a progressive vasoconstrictive oligemic anoxia. A further descriptive definition by Harkins of shock is that it is an oligemia *initiated by* traumatic local fluid loss, either whole blood, plasma, or both; *accompanied by* decreased cardiac output, diminished volume flow, lowered venous pressure, decreased oxygen consumption, arteriolar vasoconstriction, acapnia, and secondary blood pressure fall; and *perpetuated by* a summation of these factors and possibly hyperpotassemia, increased generalized capillary permeability, anoxia, action of tissue metabolites, and deficiency of adrenal cortical hormone. Other changes, both chemical and pathologic, often occur in shock, including increased blood nonprotein nitrogen, decreased coagulability of the blood, and, in some instances, increase in plasma magnesium.

Moon²² states that *hemoconcentration is the earliest detectable sign of threatened circulatory deficiency of capillary origin and it is as ominous as a decline in blood pressure*. In shock, abnormal conditions exist which permit blood plasma to escape by leakage into the tissue spaces. It can be detected by increased volume of red cells shown by hematocrit, by an increased specific gravity of the whole blood, by an increased hemoglobin content, or increased red count. A definite increase in either of these indicates hemoconcentration but the degree and significance of it cannot be estimated except by comparison with previous data. The mechanism of hemoconcentration is based upon studies of Heidenhain made some fifty years ago. He showed that the maximum flow of lymph coincided with the period of minimum arterial blood pressure. He interpreted these results as demonstrating secretory activity

*Harkins, Henry N.: *Surgery* 9: 231, 447, 607, 1941.

²²Moon, V. H.: *Am. J. Clin. Path.* 11: 361, 1941; *Shock and Related Capillary Phenomena*, New York, 1938. Oxford University Press; *Ann. Int. Med.* 13: 451, 1939; *Arch. Path.* 20: 561, 1935.

of capillary endothelium. Recent investigations, however, show that the increased flow of lymph and the accompanying phenomena including hemoconcentration are due, not to increased secretory activity, but to endothelial damage. The injection of certain substances, such as watery extracts of various normal tissues, causes a disturbance of the systemic circulation, characterized by weakness, pallor, rapid pulse, perspiration, thirst, nausea, vomiting, rapid shallow respirations, loss of tissue turgor, restlessness or apathy, low temperature, and a declining blood pressure. These are the important features in the clinical syndrome of shock. They are quite similar to the effects of serious loss of blood by hemorrhage. In shock, there is a disparity between the volume of blood and the volume capacity of the vascular bed stream, due to two causes; first, a decrease in the blood volume; and, second, an increase in the volume capacity of the vascular bed. The total blood volume is lowered by leakage of plasma into tissue spaces, and the effective volume is reduced by sequestration of blood in atonic capillaries and venules. The dilatation of these has increased their volume capacity. Furthermore, circulatory failure of capillary origin is accompanied by a characteristic group of departures from physiologic constants. The total blood volume, the volume flow, the oxygen content, carbon dioxide combining power, chloride content, and coagulability of the blood are reduced. All metabolic activities are decreased. The red cell count, hemoglobin content, specific gravity, nonprotein nitrogen, sugar, and potassium content of the blood are increased. In addition, there are visceral alterations which are seen at autopsy, which are marked diffuse capillo-venous congestion in the lungs, mucosae, and in parenchymatous organs; edema of soft visceral tissues and effusions in serous cavities; petechial hemorrhages in the lungs, parenchymatous organs, and in mucous and serous surfaces. Kidneys show parenchymatous degeneration. Liver shows severe degeneration and central necrosis. The myocardium may or may not show microscopic evidence of degeneration. These are all due to lack of oxygen. Spleen is flabby and relatively bloodless, sometimes contracted, seldom congested.

In reference to burns, the contribution of Lee, Elkinton, and Wolff²³ on "The Management of Shock and Toxemia in Severe Burns" bears consideration. They showed that a chemical study of blood from patients with extensive superficial burns showed definite hypoproteinemia concomitant with hemoconcentration. Repeated transfusions of plasma controlled these conditions because the capillaries returned to a state of normal permeability about 48 hours after the burn. At this time, the plasma volume and circulating plasma protein are returned to normal by a large transfusion of plasma. Shock, which follows every severe burn, is the result of circulatory failure which begins within a few hours and persists for two or three days after the burn. It is caused by a shift of fluid, or leakage of plasma, into tissue spaces, resulting in hemoconcentration, circulatory failure, and massive edema at the burn site. In spite of this extreme hemoconcentration, the burned patient is not dehydrated but shows an abnormal distribution of fluid and a serious impairment of the mechanism which controls its distribution. This results in a

²³Lee, Walter E., Elkinton, J. Russell, and Wolff, William A.: *Pennsylvania M. J.* 44: 1114, 1941.

paradoxical situation of concentrated, partially dehydrated blood flowing through waterlogged tissues. This condition is aggravated by large amounts of oral or parenteral fluids without improving the circulation and may lead to water intoxication. Lee et al. showed in their series that plasma transfusions were used to marked advantage and were preferred to whole blood. Small plasma transfusions were given at the sixth, fifteenth, and thirtieth hours following the burn, thus preventing a dangerous drop in blood pressure and plasma volume. By the fortieth hour, the capillaries had recovered sufficiently to hold back protein, and a large transfusion of 900 c.c. was then given. They give the following working formula to determine the amount of plasma required by ordinary cases—enough plasma should be given to keep the hematocrit value between 50 and 55 per cent cells, and the plasma protein level about 6 grams per cent until the capillaries return to a normal state of permeability. At that time enough plasma is given to restore the plasma volume to normal.

The following formula is offered to meet this requirement: Plasma protein requirement in grams =

$$3.5 W - \frac{W (100 - H_o) H_n P_o}{2 (100 - H_n) H_o}$$

W = body weight in kilograms.

H_n = normal hematocrit value, per cent cells.

H_o = observed hematocrit.

P_o = observed plasma protein.

The plasma protein requirement in grams multiplied by 14 gives the volume of plasma required, expressed in c.c.

This circulatory disturbance, of which hemoconcentration is characteristic, develops in connection with extensive trauma or surgery, burns, poisoning with certain drugs and chemicals; severe metabolic intoxication, abdominal emergencies such as perforation, strangulation, pancreatitis, mesenteric thrombosis and others, and infections of unusual severity or virulence. In traumatic or surgical shock, therefore, there is seen hemoconcentration, and leakage of plasma into tissue spaces occurs. Blalock observed hemoconcentration in dogs when shock was induced by trauma, burns, or by intestinal manipulation.

Wound shock occurs two, three, or four hours after severe tissue injury, with pain and psychic factors through their effect upon the vascular system. Wound or secondary shock is characterized by a profound fall in blood pressure with a definite reduction in blood volume, even though no hemorrhage has occurred. Hemoconcentration is a characteristic of deranged physiology seen in shock, burns, infections, and use of chemicals. There is definite loss of fluid due to damaged endothelium.

The quickest method of determining the increased volume of red cells is the hematocrit technic (pages 594 ff.). Another step in estimation of hemoconcentration is a determination of the specific gravity of whole blood. This can be done by using the falling drop densitometer. (See pages 291 ff.) This procedure can be carried out in about two minutes. The average normal value of peripheral blood is a specific gravity of 1.0566 in the male and 1.0533 in the female. The protein content may be calculated from the plasma specific gravity. These tests are adapted to an estimation of fluid imbalance, which is the predominant characteristic of cases of shock. Simple dehydration represents acute water

loss, where there is a marked concentration of cells and dehydration is shown by the rise in cell volume and protein level. Treatment of this condition is simply by administration of fluids until values are reduced to approach the normal. In shock, however, there is a much more complicated mechanism. Here, an attempt must be made to overcome the severe arteriolar and venular spasm, the capillary paralysis and dilatation, and the great loss of circulating blood volume.

In hemorrhage, there is a fall in specific gravity of whole blood, a drop in the cell volume, and a slight drop in plasma specific gravity. Volume may be restored by transfusion of whole blood but this avails little in cases characterized by shock.

In burns, there is a loss of plasma and fluids by exudation, with shock due to anoxemia from changed permeability of capillaries, when toxic substances are absorbed from burned areas. Hemoconcentration and loss of plasma into the tissue spaces of the body occur in all these cases.

In nephritis, there is an indication for the use of fluids. Without edema but with marked albuminuria, whole blood may be used if there is considerable anemia present, and plasma is indicated only when blood is normal so far as anemia is concerned. Manifestly, plasma is indicated because the high protein loss is inadequate to maintain blood plasma proteins.

In nephritis with edema, plasma finds its greatest profit and usefulness. Lack of protein in the blood is due to loss of plasma through the kidneys. Reduced colloid pressure below the edema level permits increased osmotic pressure in the extravascular spaces and the accumulation of electrolytes and water in the tissues.

In nutritional edema, the edema is due to lack of blood protein because of an inadequate protein intake. Restoration of the blood plasma proteins to normal by transfusion of whole blood or plasma causes disappearance of the edema.

It is obvious that the use of plasma possesses definite advantages over whole blood in certain cases. The preparation of plasma can be carried out under bacteriologic precautions, and the product can be safely stored for months without deterioration. *Plasma requires no preliminary typing or cross-matching.* Liquid plasma may be prepared by laboratories and stored without refrigeration. It may be carried in the physician's emergency bag and given very quickly with a special apparatus to be described later.

The treatment of shock requires replacement of lost fluid and prevention of further fluid loss. According to Harkins,* the first aim can be best attained by adequate blood or plasma transfusion. The advantages in plasma transfusions consist of safety, simplicity, preservability, and portability. It must be remembered that in addition to the replacement of lost fluid, the prevention of further loss of fluid still remains a difficult part of the treatment, and for this part of the treatment, resort must be had to oxygen and adrenal cortical extract. As Harkins states, both are still in the experimental stage but offer considerable promise, in that oxygen may act in preventing anoxia, while adrenal cortical extract may prevent increased permeability of capillary wall and cell membranes and prevent a possible hyperpotassemia.

*Harkins, Henry N.: *Surgery* 9: 231, 447, 607, 1941.

The Technic of Preparation of Plasma

This technic requires the use of equipment by which blood may be obtained and the plasma prepared under sterile conditions. The equipment of choice is that manufactured by the Baxter Laboratories.

For the preparation of plasma by this method it is necessary to have a supply of Transfuso-Vacs of 500 c.c. size, containing 50 c.c. of 4% sodium citrate solution as anti-coagulant. The bottle is a sterile vacuum unit designed to fit centrifuge cups. For the removal of the supernatant plasma after centrifugation, sterile vacuum containers are available. These are known as "Plasma-Vacs," and are half full of physiologic saline or physiologic saline and dextrose for the preparation of dilute plasma, or are empty for the preparation of undiluted plasma. In addition, it is necessary to have a Plexitron expendable set or a Transfuso donor set, which consists of a special valve and needle for the control of the blood flow into the sterile vacuum container.

Prepare the blood donor in the usual manner. Cleanse the skin at the site of the needle insertion with alcohol-iodine. Constrict the upper arm with a short piece of rubber tubing or preferably with a blood pressure cuff.

Withdraw blood in the manner outlined on pages 1093 and 1094.

Before placing the blood in a refrigerator, break the remaining vacuum with a sterile air filter.

When the Transfuso-Vacs are to be centrifuged, remove the air filter and place the bottles in centrifuge cups and balance accurately. When centrifuging such large quantities, it is imperative that the bottles opposite one another be in perfect balance. If they are not, it is not possible to prepare clear plasma, and the centrifuge is likely to be damaged. For this balancing, place rubber bands around the neck of the lighter Transfuso-Vacs until they are in balance with the heavier ones.

Centrifuge for 45 minutes to 1 hour at 2,000 r.p.m. to separate the plasma from the cells. Any jarring of the centrifuge or the Transfuso-Vacs will disturb the cell layer, and poor plasma will result.

For removal of the supernatant plasma, substitute a long blunt-end needle for the donor needle on the Transfuso donor set. Clean thoroughly and autoclave this aspiration set before use. Observe the same rules and precautions with respect to closing the valve as given on page 1094. Plunge the donor valve needle through the rubber stopper of the Plasma-Vac. *Do not flame* the aspirating needle.

Cut away the thin rubber disk.

Disinfect the stopper of the Transfuso-Vac with alcohol-iodine, and insert an air filter.

Remove the long aspirating needle from its sterile wrappings and thrust it through the thin depression in the stopper into the plasma. It is important that the needle of the air filter be above the surface of the plasma. After inserting the long aspirating needle, open the valve gently without disturbing the bottle and allow the plasma to flow into the Plasma-Vac. See Fig. 272.

After all the plasma has been removed, close the valve, and allow the small amount of plasma remaining in the tubing to run into sterile test tubes by holding the tubing vertically and opening the valve slightly. Then test the plasma samples for sterility by anaerobic and aerobic cultures. If, after sufficient time has elapsed, the plasma is found to be sterile, it is ready for use or dilution. Do not use plasma containing undue amounts of fats or showing signs of high bilirubin content.

In the preparation of accessories all distilled water used should be pyrogen-free, sterile, and otherwise suitable.

The completed pooled plasma or serum in accordance with the regulations of the National Institutes of Health relating to plasma shall contain not more than 25 mg. of hemoglobin per 100 c.c.

What is meant by **pyrogen-free** distilled water? In 1911 Hort and Penfold¹ showed that the intravenous injection of freshly distilled water caused no symptoms in animals and in man, while the intravenous injection of the same water, after it had been incubated in unsterile vessels, caused a febrile reaction. These writers decided that this principle, provisionally called *pyrogen*, was a product associated with bacteria but was not a part of the bacterial bodies themselves. It is obvious that the fever-producing principle was still present after the water had been passed through a Berkefeld filter. These facts were confirmed by Siebert in 1923² by injecting distilled water intravenously into rabbits. In 1934 Banks³ reported that *Pseudomonas urea* and *scissa*, which he isolated from surface waters, were pyrogenic. Most of the work on pyrogenic substance has been confined to the bacteria found in waters. Co Tui and M. H. Schrift in an article, entitled "Production of Pyrogen by Some Bacteria,"⁴ showed that pyrogen production is much more widespread than was first embraced in the concepts of Siebert and Banks. They investigated eighteen strains of microorganisms of different genera, tribes, and families. They made their tests in three different ways: first, with bacterial strains grown in initially pyrogen-free broths; second, they used bacteria which were most convenient to grow on slants or in Kolle flasks, washing out the colonies with pyrogen-free physiologic saline solution containing 0.4 per cent phenol, inactivating for forty-five minutes by heat, then filtering and testing the sterile filtrate; third, they tested nine organisms in the form of vaccine made with pyrogen-free water. All the vaccines were passed through a Berkefeld filter.

Their estimate of a pyrogenic reaction in a dog was, first, a leukopenia thirty to forty-five minutes after the intravenous injection of the pyrogenic material; second, a rise of temperature thirty minutes after injection, reaching a peak in from two to four hours and returning to normal in four to five hours. In mildly severe reactions there were prostration and gastrointestinal symptoms, such as vomiting and diarrhea, both of which may contain blood. These symptoms are comparable to those in man.

The Pyrogenicity of Bacterial Contaminants Found in Biologic Products

The results of investigation by Probey and Pittman⁵ are pertinent. They investigated the pyrogenic properties of those microorganisms isolated from blood or plasma during the various stages of processing of dried normal human plasma, to determine the quantitative and qualitative nature of the thermal response of rabbits (a) to the living bacteria, (b) to heat-killed bacteria, and (c) to whole cultures grown in serum. We hope that this information will be useful as a guide in determining standards for biologics intended for human therapy.

The materials used were forty-nine cultures from eight processing laboratories widely distributed throughout the United States. Twenty-eight representative cultures were selected for study, including staphylococci, micrococci, streptococci, gram-negative bacilli, and sporeforming and nonsporeforming

¹Hort, E. C., and Penfold, W. J.: Brit. M. J. 2: 1589, 1911.

²Siebert, F. B.: Am. J. Physiol. 67: 90, 1923.

³Banks, H. M.: Am. J. Clin. Path. 4: 260, 1934.

⁴J. Lab. & Clin. Med. 27: 569, 1942.

⁵Probey, T. F., and Pittman, M.: J. Bact. 50: 397-411, 1945.

gram-positive bacilli. The sodium chloride solution, which was used as the diluent, was prepared with one lot of sodium chloride proved by animal test to be pyrogen-free, and with water freshly redistilled in a glass still. Each lot was tested and accepted for use only if the rabbit thermal response induced was characteristic of pyrogen-free saline.

Methods.—The thermogenic properties of the test material were determined by the pyrogen test as described in the *U. S. Pharmacopoeia* XII, with a few changes.

The pyrexial response induced by the bacterial suspensions was computed by three procedures. The first procedure, *high above normal*, which is the U. S. P. method, bases the computation on the temperature deviation from the pretreatment normal temperature. The second method, *high above control*, records the difference between the maximum temperature deviation induced by the test material and that of the pyrogen-free control. The third procedure, *peak above control*, computes the pyrexia as the difference between the temperature induced by the test solution at the time of maximum temperature rise and the temperature of the pyrogen-free controls at the corresponding hour.

The pyrogen-free saline control in practically every instance, as originally reported by Hort and Penfold, induced a depression from the preinjection normal temperature. Therefore the normal physiologic reaction of the rabbit to injection of pyrogen-free saline is depression of the normal temperature. The fact that the pyrogen-free saline control induced a depression in the normal rabbit temperature caused these workers to believe that the evaluation of the pyrogen test should be predicated upon the normal physiologic response of the rabbit to injection with a pyrogen-free control and not upon the pretreatment normal temperature as is now recommended by the *U. S. Pharmacopoeia*. Another observation is that the quantitative characteristic of pyrogens, as reported for the bacteria studied, indicates that a slight febrile response in rabbits, of less than the 0.6° C. temperature elevation permitted by the *Pharmacopoeia* XII test, is in fact due to pyrogen contamination. This marginal amount of pyrogen may be sufficient to cause clinical reactions in humans, as Co Tui and Schrifft have observed that the rabbit is only one-third as sensitive to pyrogens as man. Co Tui² later recommended that the official test be more quantitative and suggested the use of several test doses to insure freedom from marginal pyrogen contamination. These writers believe that until the characteristics of pyrogens are more fully elucidated, it is indicated that the observation period of the animal thermal test should be continued until the maximum response has been reached or until the temperature curve has definitely entered the downward phase. Their work on contaminated blood or blood plasma during the processing of dried normal human plasma indicated that the contaminating bacteria were all capable of inducing fever in rabbits and that the count of bacteria in a contaminated blood product does not furnish an index of pyrogenicity.

The dog has a much more stable thermo-regulatory mechanism but is less sensitive to pyrogen than the rabbit. A positive test in the dog is so characteristic, however, with the additional symptoms of leukopenia, vomiting.

²Co Tui: *J. Am. Pharm. A. (Pract. Pharm. Ed.)* 5: 60-64, 1944.

and diarrhea, that it is unmistakable. For this reason, a positive test in the dog is much more significant than a negative one. To summarize, the rabbit is the better animal to test for the absence of pyrogen, while the dog is the better animal to establish the presence of pyrogen. Both animals must be used to complete the picture. Both dogs and rabbits have been used for several positive pyrogen tests without developing manifest immunity or tolerance.

The rabbit is approximately three times, and the dog at least six times, less sensitive than man to purified pyrogen from typhoid vaccine.

Test Dosages: Assuming that the above figures hold for pyrogen from other sources than the typhoid bacillus, the rabbit should receive as much as three times the maximum per kilo dose in man and the dog six times. In order to approximate the dose in man, the rabbit test dose must therefore be not less than 50 to 100 c.c. per kilo body weight. While this amount of fluid almost approximates the total blood volume of the animal, if the dose is injected in 10 to 30 minutes, the rabbit can easily take care of the plethora. But, in any event, the symptoms of plethora are different from those of a pyrogenic reaction. As the dog is to be used as a *positive* test animal, any dose which elicits the reaction shows the degree of pyrogenicity of the solution. This can be arrived at by trial and error, starting from a minimum dose of 250 c.c.

Procedure of the Test. The test is run in two parts, the preliminary part on rabbits, the confirmatory on dogs.

Preliminary Test.—Use albino rabbits of from 2½ to 3 kg. These rabbits should previously have been conditioned, for several days, to handling and taking of rectal temperatures until these procedures provoke no struggling and their rectal temperature readings are constant within 0.25° C. in three readings taken 15 minutes apart. Before injection, temperature readings taken at 15-minute intervals must not be over 39° C. The thermometers must be inserted the same distance beyond the internal sphincter of the rectum and allowed to remain there for two minutes.

The test solution is warmed to nearly the temperature of the animals; 50 c.c. per kilo body weight are injected into one animal, and 100 c.c. per kilo body weight into the second animal, no faster than 10 c.c. per minute. The temperature readings are taken every half hour for 4 hours after the injection. A rise of over 5° C. in 1 to 3 hours after injection is taken as presumptive evidence of the presence of pyrogen. A pyrogenic sample ought to give a more severe response with 100 c.c. than with 50 c.c.

Confirmatory Test: This is not necessary where a positive presumptive test is enough to lead to the condemnation of a product as unfit for human use. When more positive evidence may be required, in many legal or medicolegal complications, the clear-cut result of a positive dog test is not easy to controvert. Perform the test as follows: One healthy dog of 15 kg. body weight, conditioned to the taking of rectal temperature, with an initial rectal temperature of not over 39° C. and blood count not exceeding 20,000 white blood cells per cu. mm., is used. Depending upon the response of the rabbits, from 250 c.c. to 1,500 c.c. of the fluid are given intravenously through one of the external jugular veins, not faster than 30 c.c. per minute. The blood count is again taken 45 minutes and the temperature every half hour after the injection. The presence of leukopenia, a fall of 5,000 cells per cu. mm. or over, and temperature rise of 5° C. from 1 to 3 hours after the injection are evidences of the presence of pyrogen. The occurrence of emesis, prostration, and diarrhea are added checks.

Pyrogenic production is more common than is generally believed. It is not confined to any one group but occurs in a number of different groups of bacteria. Nor is it associated with nucleic acid, with pathogenicity, chromogenicity, nor with the property of taking the Gram stain. The production of pyrogen by a member of the yeast group suggests that even higher forms than bacteria may possess this property of pyrogen production. Chemically and immunologically pyrogen is still an unknown entity.

The importance of pyrogenic substances resides in the fact that they cause reactions in the use of improperly cleaned infusion equipment and intravenous solutions, such as Ringer's solution, glucose solution, citrated solutions of defibrinated blood, etc.

Dried Plasma*

In order to provide plasma in a compact form, resort has been made to the use of the lyophile technic, such as was worked out by Flosdorf and Mudd.¹ These two workers determined that the content of antibodies and complement of serum which is rapidly frozen and dehydrated from the frozen state under high vacuum suffer no detectable loss in the processing and, furthermore, that the rate of the subsequent deterioration is reduced to a small fraction of that which takes place in the liquid state. Scudder² stated that whole blood deteriorates rapidly, while lyophile plasma may show no appreciable change even after years. By the electrophoretic method of analysis the protein patterns for the lyophilized plasma differ little from that of the fresh control, while protein patterns dried at 37° C. show wide variation.

Method of Preparation of Dried Plasma.—

A number of methods have been described but the recommendation is made that if one desires to use dried plasma, it should be purchased from a reliable firm. 'Lyovac' is the brand of rapidly lyophilized normal human plasma prepared by Sharp & Dohme of Philadelphia. By permission of the copyright owner, Sharp & Dohme, Incorporated, as originally presented in their booklet (May, 1941) *'Lyovac' Normal Human Plasma*, the following facts and illustrations are reproduced. Blood by their process is obtained from healthy donors, who have undergone physical examinations and are found to be free of infectious disease and in good physical condition. History must be negative with respect to venereal disease and malaria. Blood is collected in bottles containing sodium citrate, using a closed system, under conditions approved by the National Institutes of Health, and a meticulous aseptic technic is rigidly exercised. Immediately after bleeding, the blood is centrifuged in the original containers, at a temperature of 2° to 4° C., for the purpose of separating the plasma from the cellular elements. Plasma from fifty bleedings is then pooled. The pooling of a large number of bleedings is carried out in order to obviate the necessity of classifying the plasma with respect to the blood group of the recipient before use. Typing is unnecessary because of two facts: first, the isoagglutinin titer of the pooled plasma is extremely low; and, second, no erythrocytes are present which might be agglutinated by the potentially incompatible serum of the recipient.

After pooling and after passing toxicity and sterility tests, 250 c.c. of plasma, representing approximately 500 c.c. of whole blood, are filled into individual containers. A sterile cotton-plugged rubber tube is then applied over the mouth of the container to maintain sterility until ready for the next

*Liquid plasma is preferred. Drying plasma preserves the hepatitis virus. Liquid plasma kept at room temperature for 6 months or longer loses its capacity to cause homologous serum jaundice.

¹Flosdorf, E. W., and Mudd, S.: *J. Immunol.* 29: 389, 1935.

²Scudder, J.: *Ann. Surg.* 112: 502, 1940.

step of the process. The container is of special design and made of a quality of glass which will withstand the rapid freezing and high vacuum to which it will be subjected.

The conversion of the liquid plasma to the frozen state is carried out at a temperature below the freezing point by immersing the container in a mixture consisting of dry ice in a suitable low-freezing solvent. By inclining the container away from the vertical position and rotating it, the material to be frozen forms a layer on the inside wall of the container; this procedure is referred to as "shelling." A large surface of the frozen material is thus exposed, which facilitates subsequent removal of water vapor when subjected to high vacuum.

Rapid freezing is highly important, for the lyophile technic aims at conversion of the substance with the least molecular rearrangement and at "fixing" of the original colloidal system.

The 'Vacule' ampul-vial, which contains the frozen plasma, is then subjected to a high vacuum in order to remove the water vapor without melting or softening the material itself. Advantage is taken at this step of the well-known physical fact that ice can be vaporized or sublimed under high vacuum without passing through the liquid phase. The necessary conditions for effecting this operation in practice are maintenance of a high vacuum by means of efficient vacuum pumps, and provision for removal of the water vapors thereby liberated.

The escaping water vapor may be removed by several methods, one of which is through the use of a condenser, the bulb being immersed in a freezing mixture so that the water is frozen therein as fast as the vacuum withdraws it from the material. The temperature of the material under process is gradually raised, after much of the moisture is removed, until it reaches approximately 20° C. or more. The dehydration is then continued until the residual moisture content is less than one per cent.

Tests have shown that the biologic activity and sterility of lyophile material are best preserved if they are maintained constantly under vacuum. For this reason the container is especially designed to maintain the desired vacuum after dehydration and is so constructed that into the neck of each container there is inserted a tightly fitting rubber stopper. This is done under vacuum after dehydration is completed. When the rubber stopper is in place, the vacuum employed in the dehydration process is released; the containers are removed from the lyophilizing apparatus and immediately flame-sealed. The vacuum, until the container is flame-sealed, is maintained by the rubber stopper in the constricted portion of the neck; the flame-sealing of the stem about one inch above the rubber stopper insures the maintenance of the existing vacuum indefinitely. The finished container, designated as a 'Vacule' flame-sealed ampul-vial, combines the best features of a glass flame-sealed ampul with those of a rubber-stoppered vial.

After a suitable interval, the finished 'Vacule' flame-sealed ampul-vials are tested to demonstrate the presence of adequate vacuum. Every container receives such inspection, and all containers which do not meet specifications are discarded. As with all biologicals, samples of the finished lyophilized blood

plasma are tested for sterility and safety. All the requirements and tests of the National Institutes of Health having been met, the product is then made available for use by the physician.

With each 'Vacule' ampul-vial of the lyophilized plasma a container of sterile distilled water is supplied. After breaking off the neck of the 'Vacule' ampul-vial at the mark just above the rubber stopper, the distilled water is transferred from the container by using the double-pointed needle which is supplied for this purpose. One end of the needle is inserted through the rubber stopper in the bottle of distilled water, which is then inverted, and the other end of the needle passed through the rubber stopper in the 'Vacule' ampul-vial of lyophilized plasma. Sufficient vacuum is present in the 'Vacule' ampul-vial to draw in all of the distilled water.

This finished lyophilized product is known as 'Lyovac' Normal Human Plasma. When desiccated it retains its therapeutic value and remains stable for at least five years and is available by the addition of pyrogen-free distilled water. It is stated that the content of specific and nonspecific antibodies, complement, and coagulating elements, together with three-fifths of the platelets, is essentially the same as that of the original fresh plasma.

Precautions Regarding the Use of This Product.—

Remove needle before all the water runs in.

Do not warm.

Dissolve in 5 minutes, shaking at room temperature, so as to obtain a clear solution.



Fig. 275.*

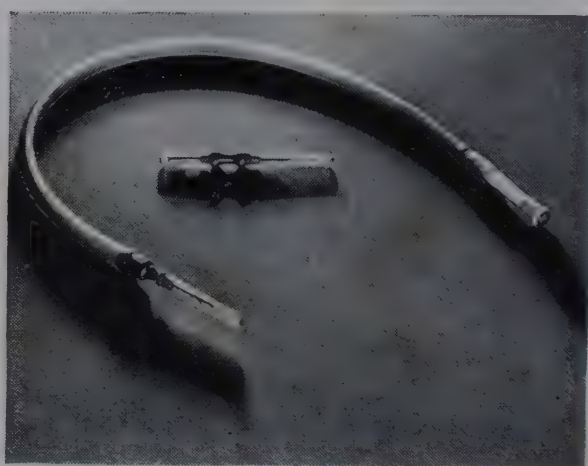


Fig. 276.

Directions for Administration of Normal Human Plasma

" 'Lyovac' Normal Human Plasma is packaged under high vacuum. Guard the container against breakage and loss of vacuum before restoration. Restore the plasma to liquid form as follows:

" Using the file provided with each package, file the neck of the 'Vacule' ampul-vial in the groove at the constriction in the neck (see Fig. 277).

" Holding the body of the 'Vacule' ampul-vial in one hand, and grasping the stem with the other hand, place the thumb directly opposite the file mark, exert outward pressure, and snap off the stem (see Fig. 278). The upper portion or 'skirt' of the rubber stopper is now

*Figs. 275 through 283 are published by permission of the copyright owner, Sharp & Dohme, Inc., as originally presented in their booklet (May, 1941) 'Lyovac' Normal Human Plasma.

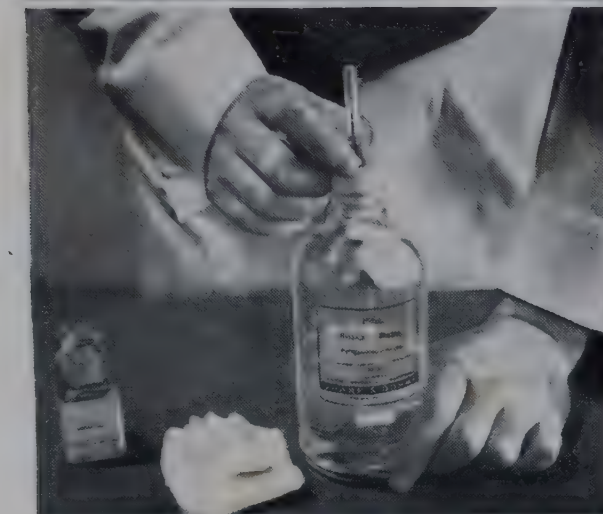
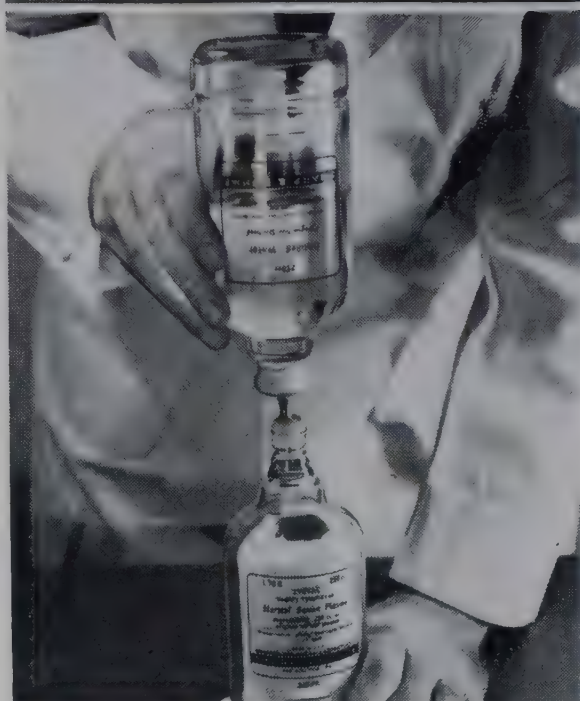
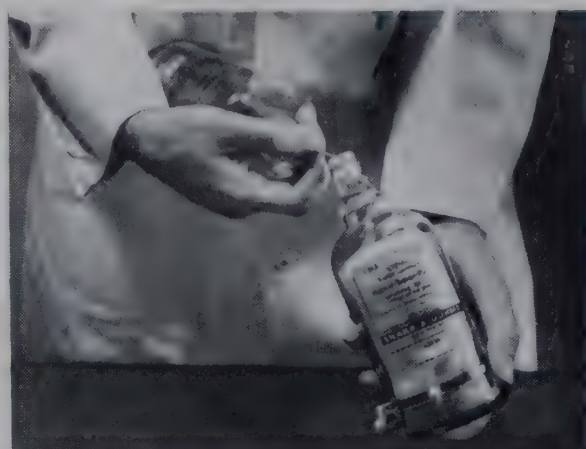


Fig.
280.

Fig.
281.

Fig. 282.

Fig. 283.

exposed and generally will automatically snap down and back to a protective position over the edges of the neck of the 'Vacule' ampul-vial. If that does not occur immediately, a slight touch of the skirt of the rubber stopper will cause it to do so.

"Remove the shorter glass cap with its rubber washer and the stylet from the double-pointed needle. Insert this exposed end of the needle through the center of the stopper in the bottle of distilled water (see Fig. 279).

"Remove the glass cap from the other end of the double-pointed needle. Hold the 'Vacule' ampul-vial of plasma in one hand, invert the bottle of distilled water in the other hand, and push the exposed end of the needle through the depression in the stopper (see Fig. 280), making certain that the water is always above the ampul-vial of plasma (see Fig. 281). There is sufficient vacuum in the 'Vacule' ampul-vial to draw in all the distilled water.

"Withdraw the empty water bottle from the end of the needle, and in a few seconds, when the vacuum has been completely satisfied, remove the needle itself. Gentle agitation will help restoration to fluid state, which is usually complete within five minutes.

"Suspend the 'Vacule' ampul-vial of restored plasma by means of the tape provided (see Fig. 282).

"Remove sterile air filter assembly from its envelope and hang the air filter end on the suspending tape. Remove the glass cap from the needle and insert it diagonally through the rubber stopper in the 'Vacule' ampul-vial (see Fig. 283).

"Prepare sterile intravenous equipment by, first, unfolding the sterile muslin wrapping, then tighten the screw clamp and the metal collar of filter and flow control. Remove glass cap and stylet from the connecting needle and insert this needle through the rubber stopper of the 'Vacule' ampul-vial diagonally and in opposite direction to the air filter needle.

"Remove the rubber cap from the sterile glass needle adapter.

"Remove cotton plug from the glass tube containing the intravenous needle. Connect the glass needle adapter to the intravenous needle while it is still in the glass tube. Withdraw the intravenous needle from tube and seat it firmly on the adapter.

"Remove the glass cap from the intravenous needle. Displace the air in the entire intravenous equipment. To do this, hold the injecting needle end of the tubing above the level of the plasma in the 'Vacule' ampul-vial, and open the screw clamp. The plasma will then run in to fill the tubing and needle. Lower the needle end of the tubing until air bubbles are expelled. Then again hold the needle end above the level of the plasma until the flow control is filled up to the mark. Again lower the needle end of the tubing to expel the last few bubbles of air and fill the tubing and needle with plasma.

"Pinch the tubing between the fingers or tighten the clamp while the needle is being inserted into the vein. When satisfied that the needle is properly in the vein, slowly release the tubing or loosen the clamp and regulate the rate of flow by adjusting the clamp. The flow control, operating in conjunction with the clamp, provides complete control of the flow rate."

CLINICAL FACTS ON PLASMA ADMINISTRATION¹

Laboratory investigation, clinical study, and the experiences of World War II have proved beyond doubt the efficacy of human blood plasma as a substitute for whole blood in the management of secondary shock. The administration of plasma as a replacement therapy in shock is based upon the idea that the oligemia and pathophysiologic cycle of shock should be corrected with an agent that increases the total circulating volume and the total circulating osmotic pressure. Hence the essence of the treatment in shock is the *"immediate, rapid, adequate replacement of the depleted blood volume with the elements lost in the proportion that they have been or are being lost."*

The plasma used during a clinical investigation was prepared from whole blood by sedimentation or centrifugation in a closed vacuum bottle system (Baxter Plasma-Vac-Centri-Vac). After transfer to the storage bottle it was

¹The author is greatly indebted to Dr. J. J. Weinstein, Associate in Surgery, Gallinger Municipal Hospital and George Washington University Hospital, Washington, D. C., for permission to use this presentation (pp. 1130 to 1138).

kept at room temperature as unpooled, diluted, or undiluted plasma without chemical preservatives. Cultures of the plasma were made at regular intervals throughout the period of storage to insure sterility. Weinstein has kept plasma on a shelf in his laboratory for five years without serious gross or chemical changes; clinical use of this plasma has proved it to be effective.

The major changes in plasma stored at room temperature (25° C.) are: (1) the precipitation of fibrin and other protein-like substances; (2) a greenish discoloration, in daylight, due to storage of plasma; (3) a rapid decrease in the prothrombin; (4) a gradual decrease in complement specific antibodies and titers of isoagglutinins. The antihemophilic component of plasma and the globulin substance, or plasma thromboplastin of Howell, are well preserved. The total protein content likewise remains practically the same as the original figures when stored for as long as two years at 25° C.

Parenteral administration is accomplished by intravenous, intramuscular, and subcutaneous routes in both adults and infants. Table 121 from a previous report indicates the methods of administration and the average amounts of plasma per injection and per individual patient.

TABLE 121.—METHODS OF PLASMA ADMINISTRATION

METHOD OF ADMINISTRATION	NUMBER OF INJECTIONS	QUANTITY FOR EACH INJECTION	TOTAL AMOUNTS GIVEN TO INDIVIDUAL PATIENTS	RATE OF ABSORPTION
Intravenous	1500 adults	250-4000 c.c.	250-36,000 c.c.	immediate
	66 infants	50- 150 c.c.	50- 2,800 c.c.	immediate
Subcutaneous	200 adults	500-1000 c.c.	500-24,000 c.c.	3- 9 hours
	340 infants	30- 75 c.c.	30- 500 c.c.	3-18 hours
Intramuscular	15 adults	500-1000 c.c.	500- 4,000 c.c.	5- 9 hours

The reactions and complications encountered with plasma administration have been minimal. General reports reveal that the incidence varies between 0.5 per cent and 3 per cent. In a previous report of 1500 intravenous infusions of plasma, a 1 per cent reaction rate was observed, and in no case was there a severe or fatal reaction (J. J. W.). The following outline describes the types and number of reactions:

Reactions With Plasma

1500 Intravenous Infusions

Types of Reactions	Number of Reactions
<i>Possible Agglutinating:</i>	
pains in lumbar regions, dyspnea, headache, tachycardia, and drop in blood pressure	1
<i>Allergic:</i>	
urticaria, edema of face, and slight dyspnea	1
<i>Chills and Hyperpyrexia:</i>	
mild group	
chill of 15 to 30 minutes' duration	10
fever of 100 to 102° F.	
moderate group	
chill of 30 minutes to 1 hour	2
fever of 100 to 103° F.	
severe group	
chill of 1 hour or more	1
fever of 103 to 105° F.	

The one reaction which simulated a hemolytic one occurred in a male patient after the administration of 75 c.c. of undiluted plasma at a rapid rate (approximately 200 drops per minute). The plasma was group A and the recipient group B. The titer of the plasma was 1:80. This same plasma was administered to the patient later in the day, at 80 drops per minute, without any evidence of reaction or hemolysis of the recipient's cells.

As much as 4,000 c.c. of unpooled plasma of various types have been administered at a single continuous intravenous injection and as much as 36,000 c.c. have been administered to one patient over a period of time. It is wise, however, to give intravenously 10 c.c. of 10 per cent calcium gluconate after each 500 c.c. of plasma. This should not be given in the line of the administration set used for giving the plasma.

The clinical usage of liquid plasma has been varied. However, by far the most important use is as a substitute for whole blood in secondary shock due to whole blood loss or as the agent of choice in secondary shock due chiefly to the loss of plasma. The more common conditions for which plasma has been given by Dr. Jacob J. Weinstein are listed below:

1. Acute traumatic shock
2. Surgical shock
3. Acute hemorrhage with and without shock
4. Infectious shock
5. Intestinal obstruction with and without shock
6. Peritonitis
7. Pituitrin shock
8. Burns with and without shock
9. Preoperative support for an anticipated long and traumatizing operation
10. Preoperative and postoperative hypoproteinemia
11. Obstructive jaundice with hepatic insufficiency
12. Primary and secondary hepatic disease with hepatic insufficiency
13. Blood transfusion reaction with severe shock
14. Malnutrition, hypoproteinemia and avitaminosis
15. Cardiac edema associated with hypoproteinemia
16. Nephritis with nephrotic syndrome
17. Lipoid nephrosis
18. Diabetic coma with peripheral circulatory failure
19. Premature infants with dehydration and malnutrition
20. Nitrogen balance postoperatively.

The treatment of secondary shock resulting from the activities of everyday life is one of the important fields of therapy for the practicing physician and surgeon. Traumatic shock has increased during the past twenty-five years because of industrialization, faster travel by air, land, and sea, and modern warfare. Since many victims of accidents are in a state of shock from hemorrhage and trauma, heroic and immediate treatment is needed. Plasma offers an immediately available substitute for whole blood which will help correct the oligemia and interrupt the pathophysiologic cycle. The dosage and end results with plasma in the management of secondary shock are dependent upon the severity of the injury, the degree of blood loss, and the severity, degree, and duration of the shock.

No hard and fast rule can be made to govern the dosage of plasma for all cases. The variations are frequent and it is best to individualize each patient.

Experience has taught (J. J. W.) that the initial dose of plasma for various degrees of shock may be:

mild shock	-----	300 to 500 c.c.
moderate shock	-----	500 to 1000 c.c.
severe shock	-----	1000 to 1500 c.c.
profound shock	-----	1500 to 2000 c.c.

The average dose of plasma usually required to combat traumatic shock in patients admitted to the Gallinger Municipal Hospital is 1100 c.c. In a given case, however, it may be important that three, four, or even a larger number of liters be given. It is necessary to remember that large amounts are needed for the successful treatment of traumatic shock. A point for review is the custom

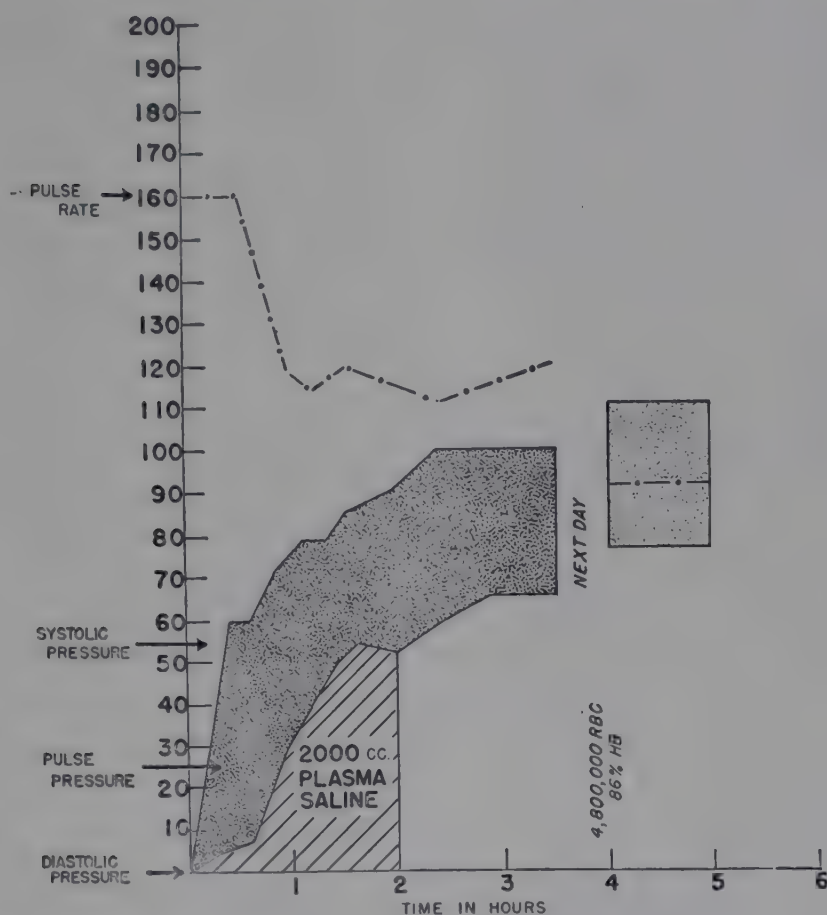


Fig. 284.—Case 1, Traumatic shock. (From White, Collins, and Weinstein: *Am. J. Surg.* 54: 701, 1941.)

of arbitrarily transfusing 250 to 500 c.c. of blood or plasma regardless of stature or the degree of shock. Did this develop because the usual amount of blood withdrawn from a donor is 500 c.c., or because the unit of plasma is 250 or 500 c.c.? Clinical experience and investigation show that this quantity has no significance. In giving plasma, it is better to err on the side of "too much too early" rather than "too little too late." However, Unger advises, particularly if the patient is bleeding externally or into a viscus, that 10 c.c. of 10 per cent calcium gluconate be given intravenously after each 1,000 c.c. of citrated blood. Otherwise the bleeding may become uncontrollable.

One of the best clinical criteria for adequate replacement therapy in secondary shock is the patient's response to the transfusion. A 15 to 25 mm. rise in systolic pressure can be expected with each pint of plasma in traumatic

shock if bleeding has stopped and no other factors are acting to produce further loss of plasma. Also if the initial dose is inadequate, give more, for no case should be regarded as irreversible until death is imminent.

Since overdosage is attended with little risk, and since inadequate amounts of plasma are wasted plasma therapy, one should always give large and repeated doses for the adequate management of shock. Fig. 284 exemplifies the importance of the immediate treatment of traumatic shock.

An interesting point for discussion in the management of shock is the rate of intravenous administration of plasma. The usual rate for the transfusion of blood is 8 to 10 c.c. per minute. The rate in shock is practically directly proportional to the degree of shock so that the greater the oligemia the greater the rate of transfusion. It is at times advisable to give 500 c.c. of plasma in 10 to 30 minutes. One of the simplest methods, in Weinstein's experience, for increasing the transfusion rate is to use a blood pressure bulb attached to the air vent of the transfusion bottle. With the screw closed, the injected air increases the pressure, thereby increasing the rate. A practical guide to the speed of injection is the clinical response of the patient. After the return of the systolic pressure to 100, the transfusion may be slowed to the conventional rate of 10 to 20 c.c. per minute until the maximal response is assured. Fig. 271 shows the Baxter pressure pump which enables administration of plasma at the normal rate or rapidly under pressure.

Surgical shock is the most common and one of the most important emergencies confronting active surgery. Results with plasma in surgical shock are not infrequently spectacular. One of the first effects of plasma upon a cold, clammy skin is the disappearance of the perspiration, and the return of warmth and color to the skin. The blood pressure response frequently follows this pattern. At first the systolic pressure rises. In fact, the systolic pressure rises more rapidly than the lagging diastolic pressure. As the pulse pressure begins to approach the normal, the diastolic pressure tends to rise more rapidly. After the functional level of 100 to 110 mm. of mercury is reached by the systolic pressure, the pulse rate may begin to decline. The pulse rate, however, is the last clinical sign to reflect the effect of treatment. It is not unusual for eight to twelve hours to pass before the pulse rate returns to normal after adequate plasma treatment of surgical shock.

Careful observations and recordings of the pulse rate in surgical patients frequently aid in the early diagnosis of secondary shock. An increase in pulse rate usually precedes the drop in blood pressure. The pulse pressure is likewise a good clinical guide to the results of therapy. The pulse pressure often directly reflects the intravascular volume. It is Weinstein's impression that the simplest and one of the most accurate guides to the detection and management of shock is the careful assessment of the pulse rate, the blood pressure, and the pulse pressure. Frequent determinations of the hematocrit, total protein, and peripheral blood counts also aid in the diagnosis and treatment of secondary shock. All patients who have been treated for surgical shock should be observed for long intervals so as to detect the development of recurrent secondary shock.

The prophylactic use of plasma in early stages of surgical shock often results in an excellent response and may allow the performance of more surgery

than would have been possible if the patient were allowed to go into a moderate or severe state of shock before treatment is instituted. See Fig. 285.

Internal or external hemorrhage, if severe enough, will cause shock and therefore requires the immediate replacement of part or all of the blood lost with carefully and accurately matched, sterile, nonsyphilitic, nonmalarial blood. It was this problem of having blood available at all times that led to the development of the donor system, then the blood bank, and finally blood plasma and serum. Two factors govern the development of hemorrhagic shock: (1)

SURGICAL SHOCK
GASTRIC RESECTION

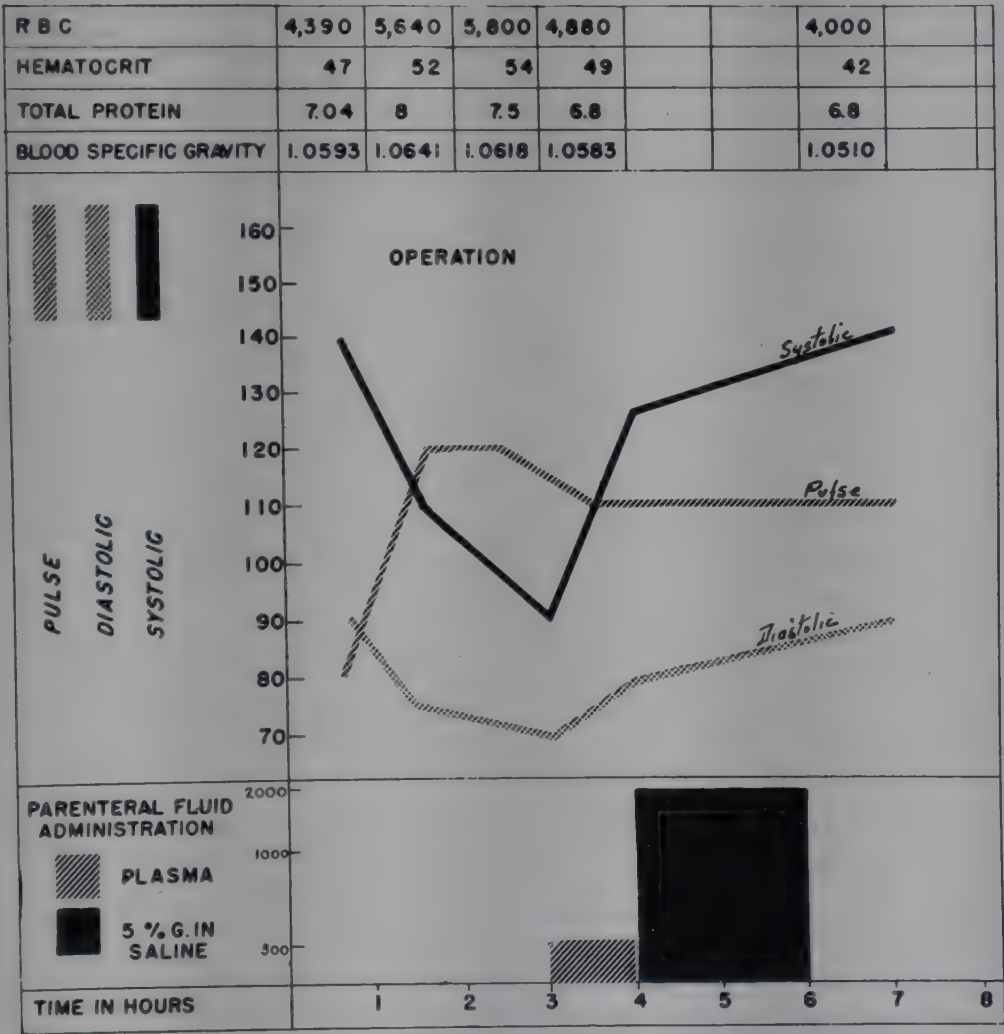


Fig. 285.—Surgical shock. (Courtesy White, Collins, and Weinstein.)

amount and rate of blood loss or lost and the amount which continues to be lost; (2) the degree and power of the compensatory mechanism to compensate for the oligemia. True secondary shock develops from hemorrhage when the oligemia is present for a period long enough to produce peripheral stagnation and capillary permeability. It is the opinion of Brodin and Saint Gerons that the major danger of hemorrhage is the loss of blood volume and that the loss of red cells is secondary to this. An acute loss of 20 to 25 per cent of the total circulating blood volume will produce shock. The loss of 50 per cent of the red blood

cells, however, will not cause death or shock if the vascular volume is adequately maintained by plasma or serum. Experience has taught that plasma is an excellent substitute for whole blood in the management of hemorrhagic shock. Plasma is not the agent of choice for hemorrhagic shock—blood is. However, the early, adequate administration of plasma in hemorrhage will prevent the development of a critical oligemia and will frequently carry the patient over until compatible blood is available.

HEMORRHAGE WITH SHOCK

NORMAL DELIVERY - ATONIA OF UTERUS
WITH SEVERE ACUTE HEMORRHAGE

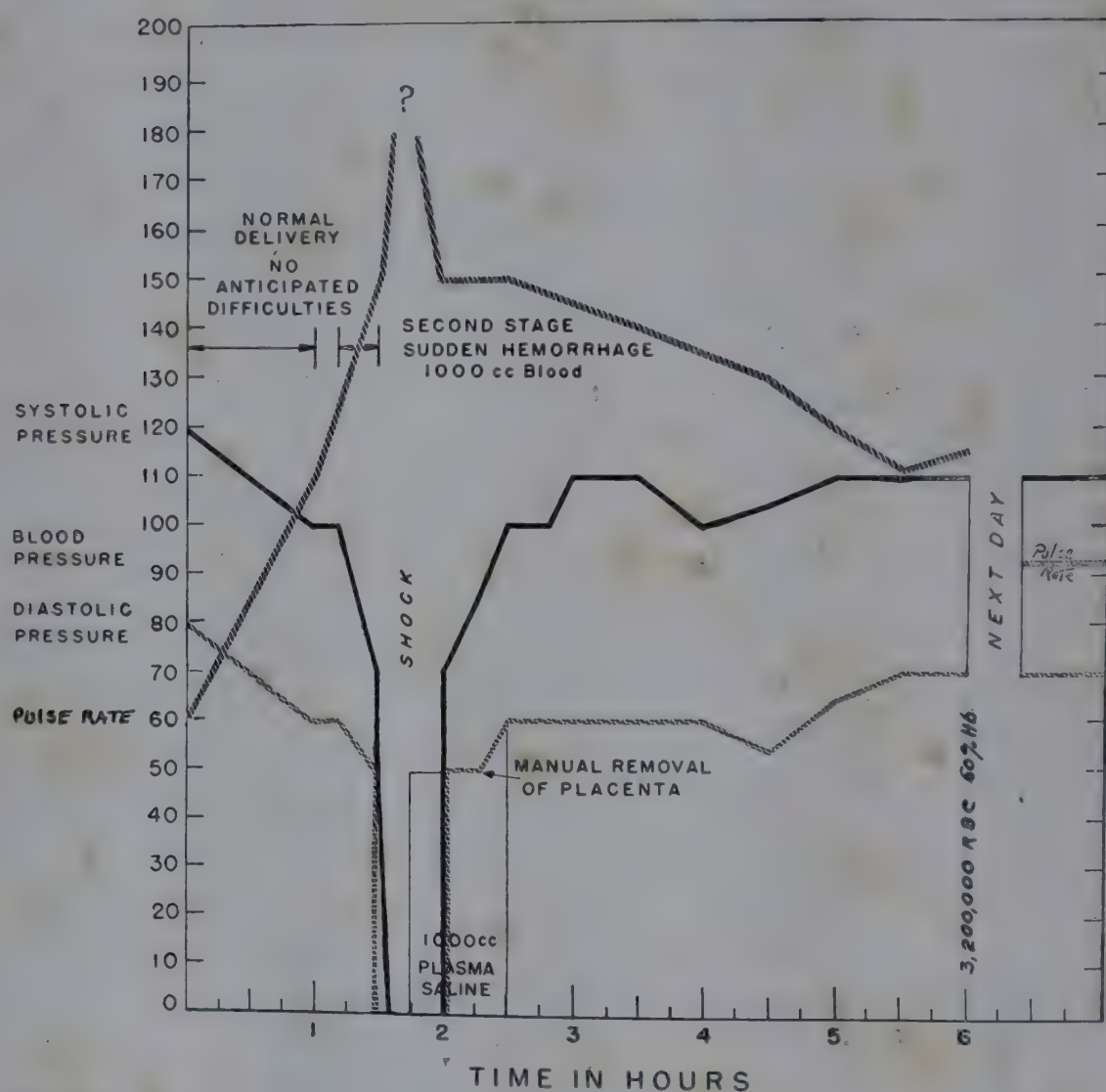


Fig. 286.—Hemorrhage with shock. (Courtesy White, Collins, and Weinstein.)

A point of caution is the confidence we may place in the effect of large doses of plasma in cases of massive hemorrhage with trauma, in those who must undergo surgery and further blood loss. These patients should be given blood and not plasma, for the continued loss of blood during surgery and the hypoxia produced by the anesthesia may produce unfavorable results. A simple rule for the administration of plasma and blood in the management of hemorrhage is to give one-half as much blood as plasma. Although plasma is not blood and one cannot expect the same results when transfusing plasma as are attained in whole blood transfusions, iso-osmotic plasma is the best blood substitute available today.

In extensive second and third degree burns there is a massive loss of plasma. The red blood cell count may rise to 8,000,000 or more, and the protein can fall as low as 3.2 gm. per 100 c.c. of plasma in the acute phase. Thirty to 60 per cent of the plasma volume is removed from the vascular bed in the acute phase of burns. Hence, the early and adequate use of large doses of plasma is important. The method of choice for determining the amount of plasma needed is the serial determinations of hematocrit, total protein, hemoglobin, red blood cell count, carbon-dioxide combining power, and urinary excretion and analysis. The blood findings closely parallel the degree of burn shock and the results of therapy.

Various rules have been promulgated for calculating the dose of plasma needed. For example, 100 c.c. of plasma should be given for every point the hematocrit level exceeds 45; or 50 c.c. of plasma for every point that the hemoglobin is above 100. To this figure is added 100 c.c. for each gram decrease below 6 of the total protein. Enough plasma is given to keep the total protein above 5.5 or 6 gm. per 100 c.c. of plasma. Close and persistent observation of the burned patient is vital, for the acute exudative phase persists for 24 to 48 or 72 hours. A shock state may appear again and again in this early phase of burns. If the burned patient is carried through the acute phase, the chances for recovery are greatly enhanced. The toxic stage, which is closely related to tissue protein metabolism, follows the acute exudative stage. During this period hypoproteinemia, edema, and water intoxication decrease the reparative powers. The hypoproteinemia is a result of increased catabolism as evidenced by the excessive nitrogen loss in the urine, the loss of serum from the granulating wounds, and the inadequate oral intake. Careful consideration should be given to the protein and fluid balance. Plasma will supply proteins for nitrogen utilization. In fact, if large enough doses of plasma are given daily, a burned patient can be kept in nitrogen balance. In passing, it may be said that the management of burns embodies the care of shock, toxemia, hypoproteinemia, water balance, and the local wounds.

Analysis of burns with shock treated at the Gallinger Municipal Hospital gives the following figures for average amounts of plasma required to overcome the acute shock stage successfully: 2400 c.c. of undiluted plasma for each adult in severe shock; 1800 c.c. in moderate shock.

The mortality for intestinal obstruction varies between 25 and 75 per cent, and the mortality for intestinal obstruction with resection of bowel is between 50 and 90 per cent. The patient with intestinal obstruction is usually much sicker than his clinical appearance. Most of the patients admitted to the Gallinger Municipal Hospital come in some time after their first symptoms of acute obstruction. The usual time is two to five days of illness. The mortality is in great part due to the pathologic changes. Patients with intestinal obstruction lose a great deal of fluid by vomiting, lose fluid and protein because of capillary permeability, are toxic from absorption of toxic waste products, and frequently are in or on the verge of secondary shock. These patients do not tolerate long surgical procedures, but frequently the disease process requires shocking and long operations. Plasma overcomes in great part the fluid imbalance present. It prevents or inhibits the development of shock. This is particularly true where long operations are carried out. Plasma has brought successful results in many cases, whereas the use of crystalloids only would have ended fatally. Blood studies

of cases with intestinal obstruction have shown findings characteristic of peripheral vascular failure. Frequently large and repeated doses of plasma are required to obtain a positive result. Occasionally one encounters a case of long-standing obstruction in which irreversible changes have occurred in the capillaries and vital centers, so that plasma will not overcome this state. These patients go on to exitus.

Surgical hypoproteinemia is a condition which is now being more carefully watched for and treated. The surgical patient who is to require intestinal surgery, particularly gastric resections, should be carefully prepared preoperatively as to the tissue and blood protein content. Frequently the patient with an ulcer of the stomach or a carcinoma of the stomach has gone through a long period of inadequate protein intake and is in a state of hypoproteinemia. This state will be exaggerated by the postoperative course, for very few of these patients take anything by mouth for the first four days postoperatively, and then the diet is limited for at least another week. The expression, "The operation was a success but the patient died," is often due to hypoproteinemia and the failure which goes with this state.

The hypoproteinemia that exists during nephrosis, chronic infections, low-grade peritonitis, specific and nonspecific colitis, and malnutrition responds frequently and satisfactorily to repeated plasma infusions. Plasma proteins are readily utilized by the body. This accessible and simple manner of supplying extra proteins to patients is of extreme importance. The edema associated with the critical low blood level of protein usually clears after sufficient infusion of plasma. This is particularly true in nephrosis and cardiac edema due to hypoproteinemia. The results with nephrosis are often only temporary and require repeated treatment with plasma. Hepatic insufficiency is another condition that is now being treated with plasma. White and Weinstein have seen spectacular results in patients with hepatorenal failure, particularly those following operative procedures for obstructive jaundice where the jaundice has caused actual liver damage.

It has been difficult to measure in figures their results with the treatment of hypoproteinemia, hepatorenal failure, etc., because the course is usually long and varied. However, one of the writers (J. J. W.) feels that clinically these patients are doing better than before the use of plasma.

One of the most enjoyable fields of investigation has been the use of plasma in the care of premature and newborn infants. The premature infant requires delicate care, adequate food and fluid, and very little handling. This is particularly true if a diarrhea develops, which is not infrequent. Blood transfusion is a common practice with infants, but that is a rather formidable procedure. However, few of these infants require red blood cells, and fresh plasma subcutaneously or intravenously will usually supply all that is needed. Also the simplicity and ease of administration offer a very important advantage as compared to the intravenous administration of blood. The complete picture of plasma administration in infants is unknown, but experience at the Gallinger Municipal Hospital has shown a decrease in morbidity and mortality with prospects for a greater future of plasma in the care of premature and newborn infants.

The **advantages of liquid plasma** are many and may be listed below.

1. Immediate availability.
2. Obviates typing, cross-matching.
3. Safe and relatively free from reaction.
4. Eliminates the waste of out-dated blood in a "blood bank."
5. The chances for transfer of syphilis are diminished. (Serologic tests are completed before storage and administration.)
6. Administered intravenously, intramuscularly, or subcutaneously.
7. May be used in large quantities and in repeated doses without pooling.
8. Stored for long periods (one and one-half years or more) at room temperature.
9. Easily transported.
10. May be quickly and simply prepared from citrated blood by centrifugation and aspiration.
11. Given without preliminary heating.
12. The small hospital can conveniently maintain a "Blood and Plasma Bank."
13. It will prevent many cases of surgical shock.
14. The more frequent use of plasma will aid any active surgical service in the preparation and care of patients.
15. Does not destroy normal plasma-cell ratio in secondary shock.

The **indications for the use of plasma**, whether dry or in liquid form, may be summarized as follows:

Use plasma in **secondary shock without hemorrhage**.

In **secondary shock with hemorrhage**, use plasma for the immediate emergency followed by whole blood as soon as possible, because there are hemoconcentration and loss of plasma from capillaries plus loss of red cells and plasma from the body. Shock develops earlier and is more severe if the loss of blood is great.

In **hemorrhage**, with loss of both cells and plasma from the body, use whole blood or plasma until whole blood can be obtained.

In **burns**, where there is loss of plasma and fluids by exudation, with shock due to anoxia from changed permeability of capillaries when toxic substances are absorbed from burned areas, use plasma. Whole blood is not indicated. In these cases, hemoconcentration and loss of plasma into the tissue spaces of the body occur.

In **infection**, with loss of blood protein, through the kidneys, use whole blood if the hemoglobin is below normal; plasma if it is normal.

In **nephritis**—without edema but with marked albuminuria—when high protein diet is inadequate to maintain blood plasma proteins, use whole blood if there is anemia present and plasma when blood count is normal.

In **nephritis**—with edema—use plasma to replace that lost through the kidney.

In **anemia**, use whole blood.

In **blood dyscrasias**, transfusion is a palliative measure and whole blood is most satisfactory.

In **nutritional edema**, with edema due to hypoproteinemia because of an inadequate intake of protein, use whole blood or plasma.

In **immunotransfusions**, when there are large quantities of specific antibodies present in convalescent blood, which are indicated in the treatment of certain infections, use whole blood or plasma.

Recommendations for Emergencies During War:

1. Every adult individual should be grouped and his group recorded. Personnel of military establishments should have records made and should have their group letters placed upon their identification tags.

2. Civilian populations in large centers should be organized into voluntary blood transfusion services.

3. Large quantities of blood should be received into the regulation vacuum bottles and refrigerated continuously, constituting a veritable wholesale blood bank.

4. As soon as the period of time has elapsed making the use of such whole blood unsafe, blood plasma should be withdrawn from these bottles, by the method already cited, and stored in sterile vacuum bottles.

5. A central agency* might assume control of this countrywide organization with the large branches constantly supplying them with information as to the number of liters of whole blood and blood plasma on hand and ready for use.

6. In times of emergency, this central organization could forward to any concentration point any quantity of whole blood or blood plasma desired.

Summary on Usage of Blood and Plasma

The transfusion of whole blood is still an essential procedure in acute hemorrhage and in some of the blood dyscrasias. It is of interest that there has been made available by a number of biological houses a modification of the original vacuum container for transfusion purposes. This container enables the storage of whole blood until the expiration time as a blood bank unit. The plasma prepared by sedimentation may be removed by the technic previously described or the bottle may be placed in a centrifuge cup and the plasma prepared by centrifugalization, resulting in a greater yield of clearer plasma.

Regarding the question of what form of plasma is preferable, it seems undeniable that for the optimum preservation of prothrombin, fluid plasma should be maintained in the frozen state. As stated by Strumia and McGraw,¹ plasma dried from the frozen state and restored with sterile pyrogen-free distilled water or plasma stored at room temperature (about 25° C.) for even short periods shows almost complete loss of prothrombin. It has been suggested² that prothrombin may be lost in the process of drying owing to the removal of carbon dioxide with resultant increased alkalinity. Plasma preserved in the liquid form at 4° C. maintains a fair quantity of prothrombin and complement, for a period of approximately fifty days, but causes maximal

*The use of plasma prepared by the American Red Cross was one of the greatest achievements of World War II, which is now happily over. So much plasma was prepared that a vast quantity was left over after the war. This excess has been distributed to hospitals free of charge throughout the United States. This war measure now has become a notable peace measure as well.

¹Strumia, Max M., and McGraw, John J.: *J. A. M. A.* 118: 427, 1942.

²Flosdorf, E. W., and Mudd, Stuart: *J. Immunol.* 29: 389, 1935.

flocculation within a few hours. Filtration is essential and the danger of bacterial growth from a chance contamination is not eliminated. While dried plasma prevents bacterial growth and flocculation, this material, when regenerated, has lost almost all the prothrombin and a portion of the complement. It has the advantage that it permits concentration if desired, but it is of little value for hypoprothrombinemia. Noteworthy is the advantage of the frozen plasma in that it prevents bacterial growth and flocculation and at the same time insures almost complete preservation of all specific elements, including prothrombin and complement. If properly thawed, it does not have to be filtered and the therapeutic value is practically equal to that of freshly prepared plasma. Thawing must be rapid in the water bath at 37° C., and thereafter, until administered, the liquid plasma must be kept at room temperature. It is the opinion of Strumia and McGraw that plasma maintained in the frozen state and properly thawed is almost identical to the original material.

Whole blood is best for pure blood loss. Plasma is better for shock, burns, and hypoproteinemias. Liquid plasma, when fresh, is best of all. When it must be stored, it is best stored in a frozen state because it retains its prothrombin and complement intact. Possibly the use of dried plasma is the most practicable of all forms of plasma preparation for large-scale operation.

AGGLUTINOGENS M AND N

Landsteiner and Levine¹ showed that when certain immune sera from rabbits which had been injected with human blood were absorbed with certain samples of human blood, they still contained agglutinins acting on the majority of bloods of all four groups, while other bloods were not agglutinated. Two of the factors demonstrable by these sera were designated M and N. These writers found that according to the content of agglutinogens M and N in blood cells, three distinct types of human blood could be distinguished: type M, which contains agglutinin M but lacks agglutinin N; type N, which contains agglutinin N but lacks M; and type MN, which contains both agglutinogens, M and N.

The distribution of these types is the same in each of the four blood groups, so that the agglutinogens M and N are unrelated to the agglutinogens A and B. Of 1,256 blood specimens, we* found 270, or 21.5 per cent, type M, 222, or 17.7 per cent, type N, and 764, or 60.8 per cent, type MN patients.

It is interesting to note that no agglutinins for N have been shown in human sera, and in only seven cases have agglutinins for M been found.² The testing sera for differentiating agglutinogens cannot be obtained as A and B antisera are, by venous puncture from human sources, but must be prepared artificially by immunizing rabbits. In order to produce anti-M serum, one must inject blood of type M (from group OM) into rabbits; one must inject blood of type N (group ON) into rabbits to produce anti-N serum.

Method of Producing Anti-M and Anti-N Sera†

First, find OM and ON subjects to furnish blood for the immunization of rabbits. Observe all sterile precautions throughout the entire procedure.

*Gradwohl Laboratories.

†Wiener, Blood Groups and Blood Transfusion and Wiener, Zinsher, and Selkova, J. Immunol. 28: 431, 1934.

¹Proc. Soc. Exper. Biol. & Med. 24: 600, 941, 1927; J. Exper. Med. 47: 757, 1928.

²Unger, Wiener, and Sonn: Am. J. Clin. Path. 16: 1, 1946.

Withdraw 50 c.c. of blood OM and 50 c.c. of blood ON directly into their respective 50 c.c. Rockefeller centrifuge tubes, each containing 4 c.c. of 3.8% sodium citrate solution. Shake to prevent clotting.

Wash with sterile saline (see page 2043) several times until all plasma has been removed. This prevents possible anaphylaxis and production of antibodies for human serum globulin. Restore to original volume. (Twelve c.c. of this blood added to 48 c.c. of saline furnishes sufficient material to inject four rabbits six times each.) It is well to pool several OM bloods for injecting the M rabbits and several ON bloods for injecting the N rabbits.

Use four rabbits* for the production of anti-M serum, and four* to produce anti-N serum. Each rabbit must be identified by an ear tag, and complete records kept. Inject into the marginal ear vein of each rabbit 2 c.c. of blood, using four of the rabbits for injection with M cells and four for injection with N cells. Repeat the injections daily for six days.

Allow one week's rest interval. Repeat the six injections, giving the first intramuscularly and the other five intravenously. Giving the first injection intramuscularly prevents anaphylaxis and possible agglutination of the cells and death of the rabbit.

Allow another rest interval of one week. Repeat the six injections, giving the first intramuscularly and the other five intravenously.

Wait another week and test to determine whether or not anti-M or anti-N agglutinins have been produced in any of the animals. The entire period of injections and intervals covers six weeks.

It will be found that not all rabbits will react well and produce an efficient agglutinating serum. Sometimes only one rabbit out of the four will develop agglutinins. For this reason it is necessary to test the serum of each rabbit for agglutinating substances before exsanguinating the rabbit from the carotid artery.

Preliminary Tests of M and N Antibodies.—Withdraw 2 c.c. blood from the ear vein of each rabbit, place in a clean, dry, sterile Kahn tube, properly labeled with the type letter and the number of the rabbit. Allow to clot. Loosen the clot, centrifuge, withdraw the supernatant serum, and place it in a clean, dry, sterile Kahn tube properly labeled. Inactivate at 56° C. for thirty minutes to prevent subsequent hemolysis and to inhibit the growth of any contaminating bacteria.

Since the sera of immunized rabbits contain species agglutinins, it is necessary to remove these before any tests can be performed. These are removed by absorption. To absorb anti-M serum, N¹ cells are added to the rabbit serum; to absorb anti-N serum, M² cells are used. Since the M serum is not easily overabsorbed while the N serum may be overabsorbed, greater care must be used in absorbing anti-N sera than is the case with anti-M sera. Absorptions are usually carried out at room temperature, using sufficient quantities of absorbing blood to insure complete removal of the species antibodies. If the absorptions are carried out at lower temperatures, smaller amounts of blood are required. Caution must be exercised in the preparation of the anti-N fluid so as to avoid overabsorption with M absorbing blood.

Dilute the serum 1:20 by adding two drops of serum to thirty-eight drops of saline. This gives 2 c.c. of fluid, representing a dilution of 1:20.

Place 1 c.c. in a Kahn tube.

Add 0.5 c.c. of washed cells to the serum. If preparing an anti-M serum, add 0.5 c.c. N cells, mix thoroughly, allow to stand one-half hour at room temperature, centrifuge, and use

*Four or more rabbits are used because many rabbits do not develop the antibody. Usually out of four rabbits perhaps only one will develop an anti-M or anti-N antibody sufficiently active to be useful in tests.

¹Pooled groups ON, AN, and BN cells.

²Pooled groups OM, AM, and BM cells.

the supernatant fluid. If preparing *anti-N serum*, use M cells, placing the tube in a glass of warm water in the incubator at 37° C. for thirty minutes, centrifuge, and use the supernatant fluid.

Test each serum against several specimens of M, N, and MN bloods. On a slide containing three wax rings, properly labeled, place one drop of M cells in the first ring, one drop of N cells in the second ring, and one drop of MN cells in the third ring. Add to each one drop of anti-M testing fluid. Rotate and examine periodically under the low power of the microscope for seven to ten minutes. Prepare a similar slide but use anti-N testing fluid instead of the anti-M. The sera show proper reaction provided (1) the anti-M serum agglutinates all M and MN cells within one or two minutes, but fails to agglutinate N cells after ten minutes; and (2) the anti-N serum agglutinates all N and MN cells in one or two minutes, but fails to agglutinate M cells within ten minutes. If the M fluid agglutinates N cells, or if the N serum agglutinates M cells, the serum must be re-absorbed, care being exercised not to overabsorb. If the anti-M serum fails to agglutinate M cells, or if the anti-N serum fails to agglutinate N cells, then the serum cannot be used.

Titrate the fluid, using the technic on page 1015. The M fluid should have a titer of at least 1:20. The N fluid need not be of a titer higher than from 1:8 to 1:16.

Collection of Anti-M or Anti-N Testing Fluids.—Having selected the rabbits which show agglutinin anti-M in a titer of at least 1:20, and the anti-N in a titer of at least 1:8 or 1:16, exsanguinate them by severing the carotid artery, using utmost sterile precautions, and obtaining the entire content of blood from each animal. For the method of bleeding rabbits from the carotid artery, see page 2040.

Collect the blood in clean, dry, sterile 50 c.c. Rockefeller centrifuge tubes, properly labeled. Cork immediately and allow to clot in a slanting position. Strike the tubes against the palm of the hand to loosen the clot. Refrigerate for twenty-four hours. Centrifuge at high speed for 15 to 30 minutes to separate the serum from the clot. Withdraw the serum, under sterile conditions, placing it in sterile 20 c.c. test tubes, properly labeled, and stoppered with cotton, and inactivate at 56° C. for 30 minutes. Save a few c.c. for absorption. Make ampules of the remaining serum, labeling with the rabbit's number, the type letter, date, and the notation "inactivated, unabsorbed." The serum will remain in good condition for a longer period if it is unabsorbed. Absorb only enough for several months' use.

Preparation of Testing Fluids Anti-M and Anti-N.—The species agglutinins from these two collections of blood from M and N rabbits must be treated as in the preliminary experimental work detailed above to remove species agglutinins. Under strict sterile conditions, dilute each serum to 15 to 25 times its volume with sterile physiologic saline and mix with the appropriate blood cells in order to complete absorption.

Absorption of M Serum.—Use all sterile precautions. Dilute this serum 1:20 with sterile saline. Divide it into two parts, each tube containing 10 c.c. It is better to handle small quantities of serum at a time. Place 5 c.c. of pooled, washed, packed N¹ cells in each M diluted serum. Cork each tube of serum, and mix by inverting back and forth about six times. Allow to stand 30 minutes. Centrifuge and test for the presence of anti-M and N agglutinins. This absorption is carried out at room temperature. Absorption is complete in the case of anti-M fluids when there is no longer a reaction with N bloods, but the anti-M serum must still agglutinate M and MN cells within a two-minute period.

Absorption of Anti-N Serum.—In the preparation of N fluids, great care must be exercised to prevent overabsorption. The preparation of N fluids is carried out slowly so that one may approach the end point of complete absorption very accurately. Place the anti-N tube, in which the anti-N serum has been in contact with the M² cells, in a glass of warm water (37° C.) in the incubator and allow it to stand the same length of time as in the case of the M fluid, namely, 30 minutes. Remove from the incubator and test. If it is not completely absorbed, add more cells, using only half the above quantity of M cells, and repeat the same technic in the incubator. This serum must agglutinate N and MN cells within two minutes but must not agglutinate M cells within a five- to ten-minute period.

Assuming that the M cells are no longer agglutinated by the anti-N serum, and the N cells are no longer agglutinated by the anti-M serum, the two products may be bottled. A small

¹Pooled groups ON, AN, and BN cells.

²Pooled groups OM, AM, and BM cells.

amount of Merthiolate, one part of a 1% solution to 100 parts of testing fluid, are added, giving a 1:10,000 concentration of Merthiolate.

Preservation of Anti-M and Anti-N Agglutinins With Toluol.—Levine¹ recommended toluol to preserve anti-M and anti-N agglutinins in testing fluids. One drop of toluol is added to each cubic centimeter of diagnostic fluid; this is then stoppered and sealed with paraffin. After thirty-two months, he found the anti-M just as active as the fluid freshly prepared from the same serum. Anti-N gave reactions which were only slightly less intense than those with the freshly prepared fluid. The best way to preserve antisera, however, is in the frozen state without the addition of any chemical.

Method of Using Anti-M and Anti-N Testing Fluids.—The preferred method is the centrifuge method. By mixing a small amount of cells with a drop of serum and centrifuging, the centrifuged cells at the bottom of the tube will not disperse when shaken if they contain the specific agglutinin. The mass of cells remains as a single large clump. If they break up into a homogeneous suspension, as seen under the microscope, the reaction is negative. The slide method may be used but for exact work the centrifuge method is preferred.

Technic of Open Slide Method of Testing for M and N Agglutinogens

Controls.—Have on hand suspensions of known type M, N, and MN cells. Use two slides with three wax rings each, properly labeled. Place one drop of M cell suspension in the rings labeled “M,” one drop of N cells in the “N” rings, and one drop of MN cells in the “MN” rings on each side. On the first slide, labeled “anti-M” serum add 1 drop of anti-M testing fluid to each ring. Add 1 drop of anti-N testing fluid to each ring on the second slide labeled “anti-N” testing fluid. Rotate and examine every 30 seconds under the low magnification of the microscope, recording a positive reaction when it occurs, and waiting at least five to ten minutes before recording a negative reaction. A positive reaction is agglutination of the erythrocytes; a negative reaction is lack of agglutination.

The M serum should agglutinate M and MN cells, but not N cells, while the N serum should agglutinate N and MN cells, but not M cells. If the controls indicate that the sera are in good condition, tests may be made on unknown bloods.

Technic of Testing Unknown Blood.—Place one drop of unknown cell suspension in each of two wax rings on a glass slide. The left-hand ring should be marked “M” and the right-hand ring “N.” Add 1 drop of anti-M testing fluid to the left ring and one drop of anti-N testing fluid to the right ring. Rotate and examine under the microscope periodically, recording agglutination when it occurs, but waiting at least five to ten minutes (as determined by the controls) before recording lack of agglutination. (Cell suspensions are described on pages 1005 and 1006.)

UNKNOWN CELLS + ANTI-M SERUM	UNKNOWN CELLS + ANTI-N SERUM	TYPE OF UNKNOWN
+	+	MN
+	-	M
-	+	N

+ = agglutination
- = no agglutination

Read the reactions as follows: If both sides show agglutination, unknown is MN. If the anti-M serum agglutinates the cells, but the anti-N serum does not, the unknown is type M. If the anti-N serum agglutinates the cells, while the anti-M serum does not, the type of the unknown is N.

If neither side shows agglutination, the sera must be discarded and new sera obtained.

¹Levine, Phillip: J. Lab. & Clin. Med. 26: 866, 1941.

In working with M and N testing fluids, as shown by Landsteiner and Levine, bloods containing factor **N** alone are more strongly agglutinated by anti-**N** testing fluids than bloods containing **M** and **N** together. Type N blood is twice as sensitive as type MN blood to agglutination with anti-**N** serum.* It must be emphasized that control bloods of all three types are necessary in the performance of M and N tests. Wiener has emphasized that in a large experience, using three different anti-**M** fluids and three different anti-**N** fluids, he has never found a discrepancy among the reactions. There are certain sources of error, however, which Wiener has outlined, which must be taken into account. First, great care must be used in the preparation of anti-**M** and anti-**N** testing fluids. *Overabsorption* is the fault of most beginners in making these fluids. He has shown that there may be interfering reactions caused by specific agglutinins other than those directed against M and N. Similar interfering reactions may be caused by natural or immune agglutinins against A and B, and there may be a coexistence in the same serum of more than one of the agglutinins against M and N. To obviate these difficulties, the preparation of the testing fluid by absorption should be carried out in the case of anti-**N** fluid by using a mixture of bloods of types OM, AM, and BM. Again, Wiener has shown that there is danger of false positive reactions from *underabsorption* of the immune sera. This particularly applies to the tests for N but may be prevented easily by the inclusion of negative control bloods. Errors are more likely to occur in using the slide method than the centrifuge test-tube method.

BLOOD GROUPS AND HEREDITY

For the practical application of blood transfusion, heredity is of importance because with a knowledge of the formulas of heredity we can understand that relatives are suitable for blood transfusions only when they possess compatible blood groups.

The serologic differences in the blood cells have been proved to be determined by heredity and are not influenced by environment. Ottenberg and Epstein¹ and von Dungern and Hirszfeld² proved that the four Landsteiner blood groups are inherited. Bernstein³ in 1925 proved the exact mechanism of heredity. These inherited factors follow Mendel's laws. With respect to what we mean by "Mendelian dominants," one must turn to the painstaking research of Johann Mendel, born in 1822, of German parentage, in the village of Heinzendorf, in Silesia. At the age of 21, in 1843, he entered the Augustinian Monastery at Brunn, Austria, there spending the remainder of his life except for a short period of study at Vienna. His name upon entrance to the Monastery became Gregor, and now he is known as Gregor Johann Mendel. In a little strip of garden behind the monastery building, Mendel conducted his experiments upon hybridization. In his rooms he hybridized mice, along the edge of the garden he experimented with bees, and in the garden itself, he crossed many kinds of plants. His famous experimental work was per-

*Wiener, Zinsher, and Selkove, J. Immunol. 27: 431, 1934.

¹Trans. New York Path. Soc. 8: 187, 1908.

²Ztschr. f. Immunitäts 6: 284, 1910.

³Ztschr. f. indukt. Abstammungs. u. Vererbungs 37: 237, 1925.

formed with garden peas, columbine, snapdragons, slipperworts, pumpkins, four-o'clocks, beans, etc. His work with garden peas is the most notable. Seven pairs of peas with contrasting characteristics were used by him, including smooth seeds and wrinkled seeds, yellow cotyledons and green cotyledons, inflated pods and constricted pods, yellow pods when unripe and green pods when unripe, flowers in the axils of the leaves and flowers at the ends of the stems, white seed coats and brown seed coats, tall plants and dwarf plants.

Mendel crossed tall plants with dwarf plants and produced the first filial generation, known as F_1 , all of which were tall. When these tall plants were selfed, they produced both tall and dwarf plants, there being three times as many tall plants as dwarf plants. In the second generation, two-thirds of the tall plants when selfed produced tall and dwarf plants in the ratio of 3:1, just like the F_1 plants. All the F_1 plants were tall, because, according to Mendel, the factor determining tallness was *dominant* to the factor for dwarfness. When hybrid tall plants were selfed, tallness showed up in the proportion of 3:1. In general, he found a factor which he called a *dominant* factor. This showed up always in the proportion of 3:1, while the factor which did not produce its effect in the presence of the contrasting factor he called *recessive*. Thus, *tallness* was the dominant factor and *dwarfness* was the recessive.

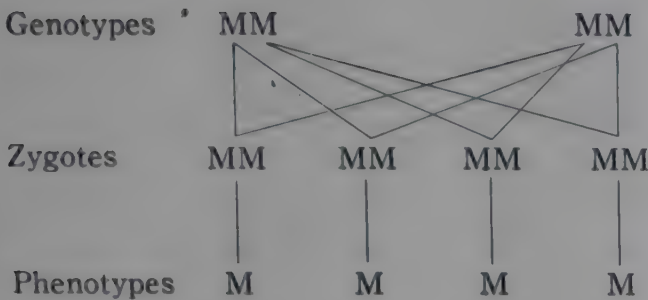
Mendel's work was published in 1866, but very little attention was paid to it until the botanists deVries, Correns, and von Tschermak reached conclusions similar to his, but independent of each other. His paper was then rediscovered and credit given to him after thirty years. Thus, in 1900, his work was made known to the scientific world.

The explanation of the heredity of blood groups may be found by studying the chromosomes of cells. There are 48 chromosomes in the human cell, or 24 pairs; in each cell of a mosquito there are 6 chromosomes, or 3 pairs; in each cell of a corn plant there are 20 chromosomes, or 10 pairs; in each cell of a certain species of crayfish, 200 chromosomes, or 100 pairs, etc. Within the chromosome, there are factors known as *genes* which are transmitted in a hereditary sense. Two such factors forming a contrasting part are said to be *alleles*, with an adjective formed from this word, *allelic*. Classifications based upon genetic formulas are known as *genotypes* and the final hereditary result is the *phenotype*. Thus in group A, we would call AA the genotype and A the phenotype; in group B, BB the genotype and B the phenotype; in group O, OO the genotype and O the phenotype, etc.

A germ cell (sperm or ovum) is called a *gamete* and the union of two gametes is called a *zygote*. A zygote is said to be a *homozygote* if the two members of the pair of factors are alike, and a *heterozygote* if the two members are different. Thus, AA is a homozygote and AO , a heterozygote. In the blood groups, A and B may be assumed to be pure strains from a genetic standpoint; that is, the genotype may be AA and BB . If it is as follows, AO or BO , A is dominant and O is recessive, so that the phenotypes corresponding to AO and BO are A and B, respectively.

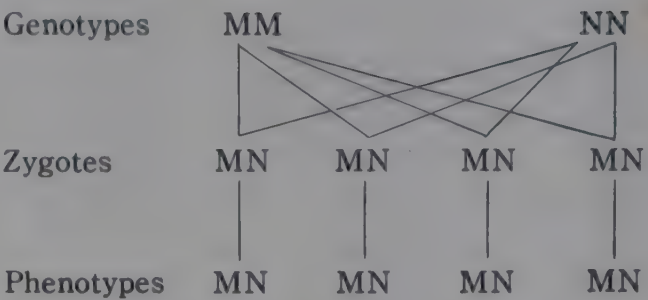
The following diagrams illustrate transmission of hereditary group factors: mating, parents' genotypes, zygotes, and phenotypes of children with respect to blood groups A and B, blood types M and N, and the Rh factor. For a fuller discussion of the heredity of the Rh factor refer to pages 1048, 1052 to 1054.

Parents: M & M



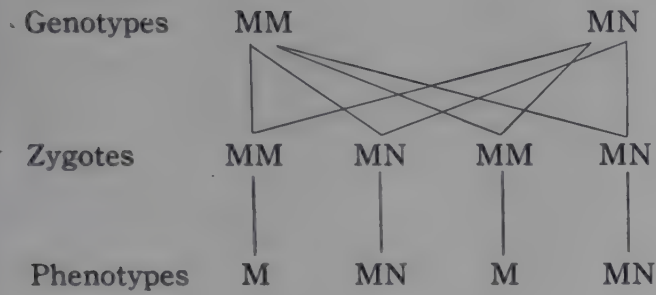
Children possible: M
Children not possible: N-MN

Parents: M & N



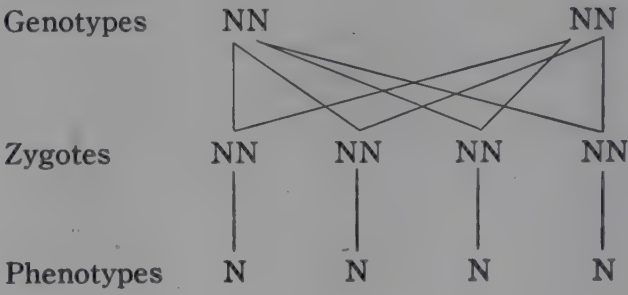
Children possible: MN
Children not possible: N-M

Parents: M & MN



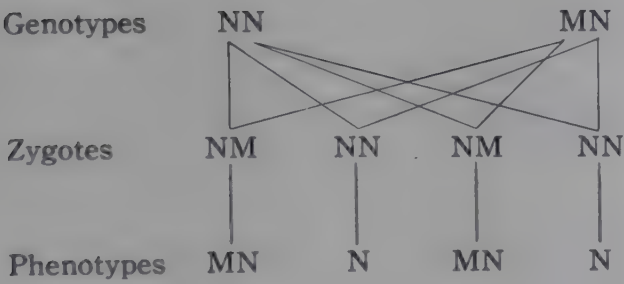
Children possible: M-MN
Children not possible: N

Parents: N & N



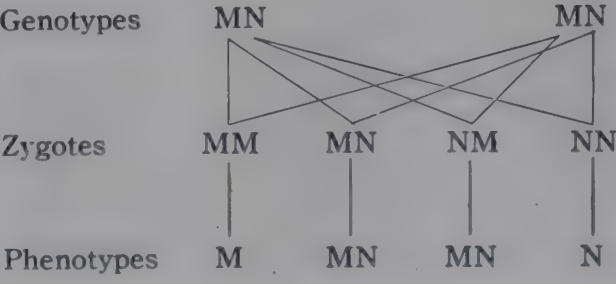
Children possible: N
Children not possible: M-MN

Parents: N & MN

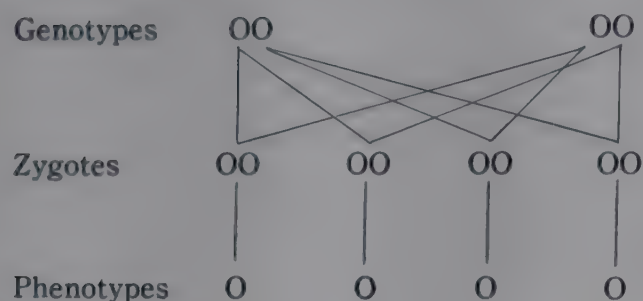


Children possible: N-MN
Children not possible: M

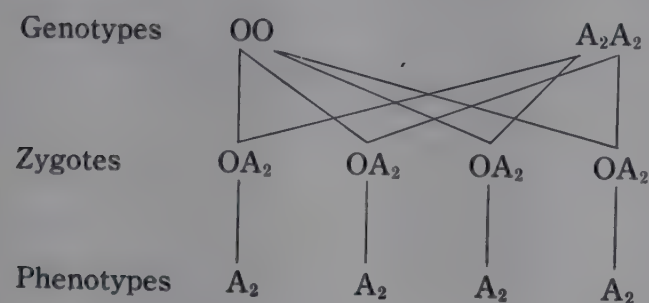
Parents: MN & MN



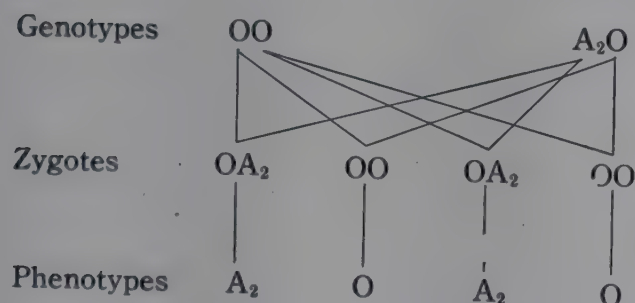
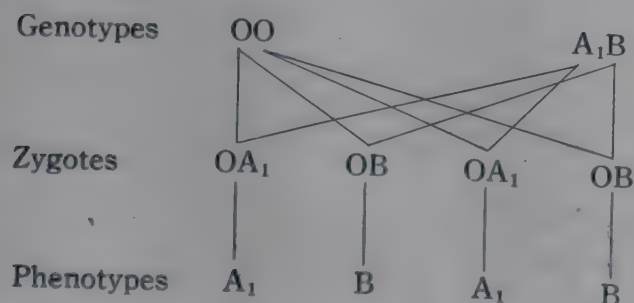
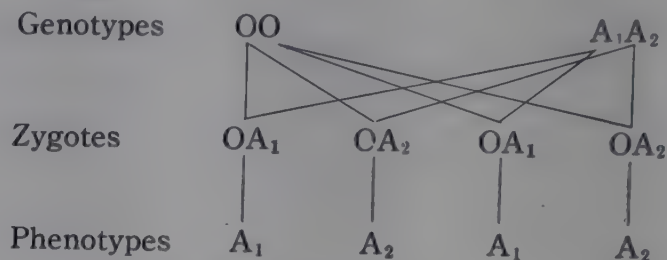
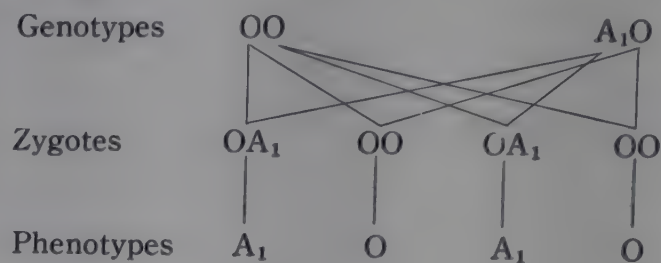
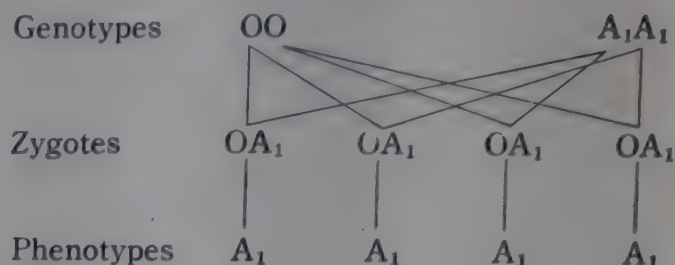
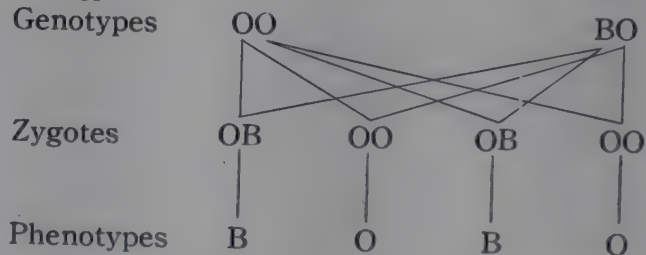
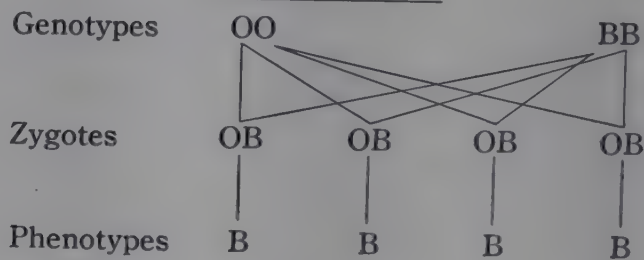
Children possible: M-N-MN
Children not possible: —

Parents: O & O

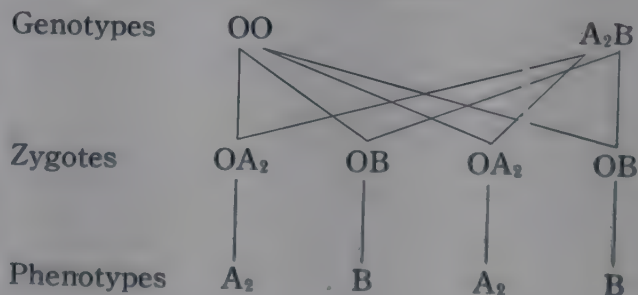
Children possible: O

Children not possible: A_1A_2 -B- A_1B - A_2B Parents: O & A_2 

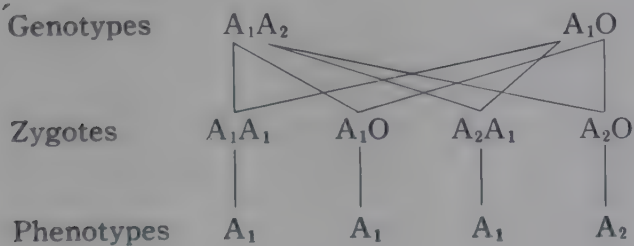
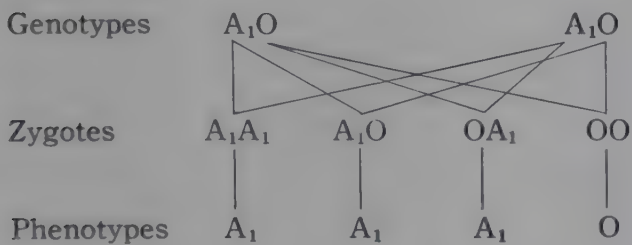
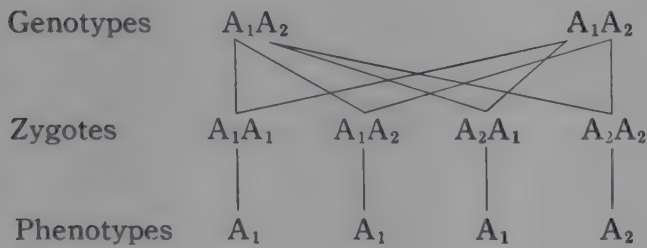
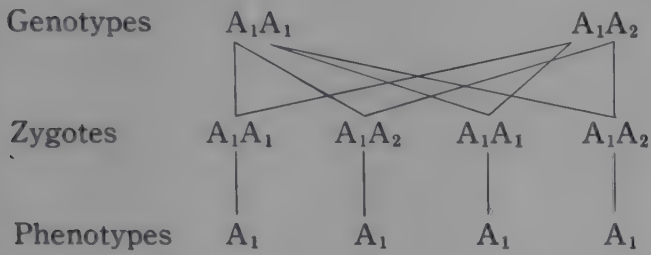
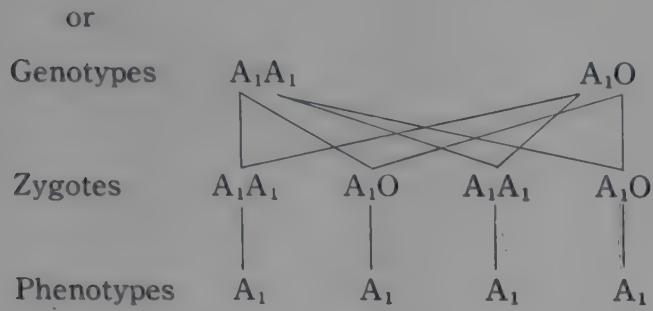
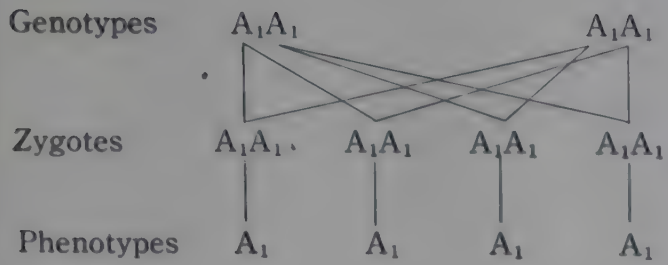
or

Children possible: O- A_2 Children not possible: A_1B - A_1B - A_2B Parents: O & A_1B Children possible: A_1 -BChildren not possible: O- A_2 - A_1B - A_2B Parents: O & A_1 Children possible: O- A_1 - A_2 Children not possible: B- A_1B - A_2B Parents: O & B

Children possible: O-B

Children not possible: A_1 - A_2 - A_1B - A_2B Parents: O & A_2B Children possible: A_2 -BChildren not possible: O- A_1 - A_1B - A_2B

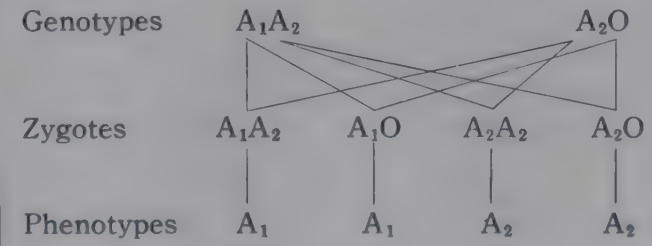
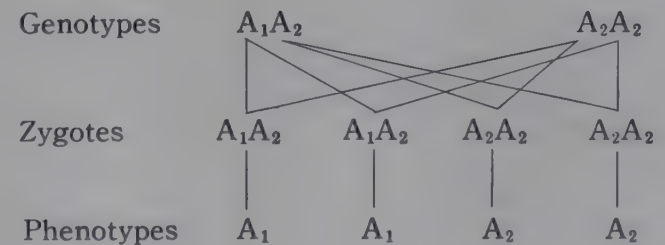
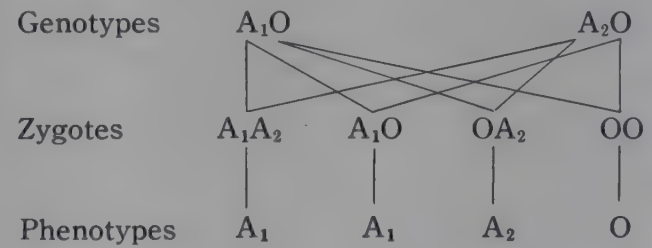
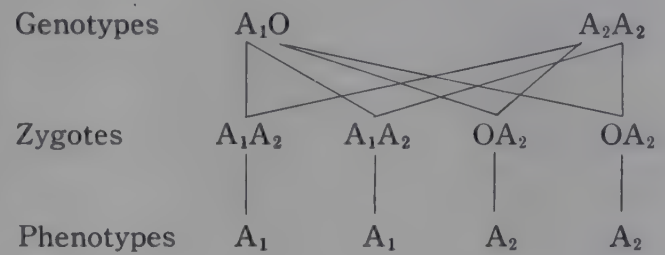
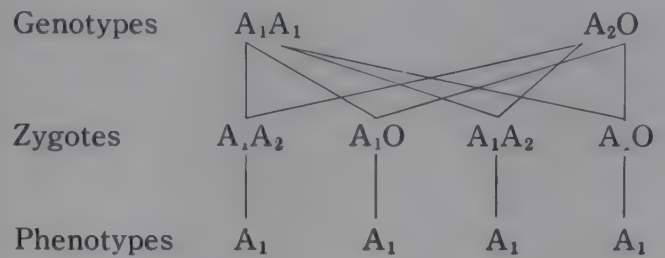
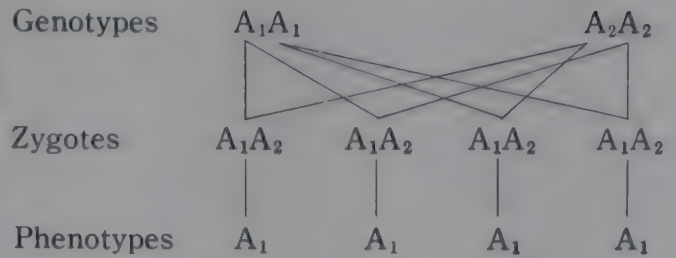
Parents: A_1 & A_1



Children possible: $O-A_1-A_2$

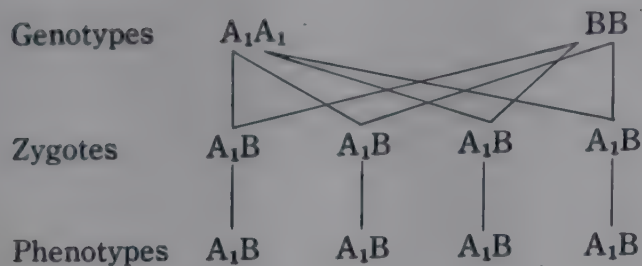
Children not possible: $B-A_1B-A_2B$

Parents: A_1 & A_2

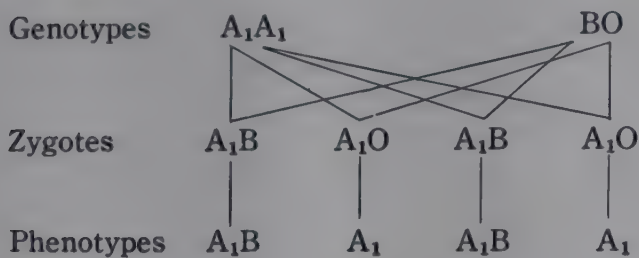


Children possible: $O-A_1-A_2$

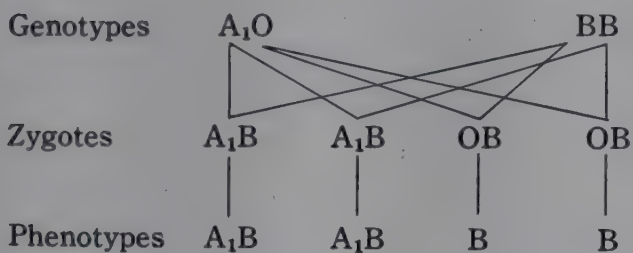
Children not possible: $B-A_1B-A_2B$

Parents: A_1 & B

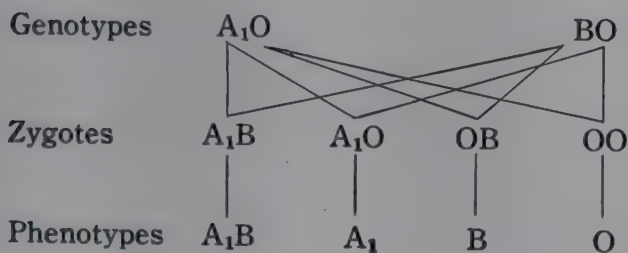
or



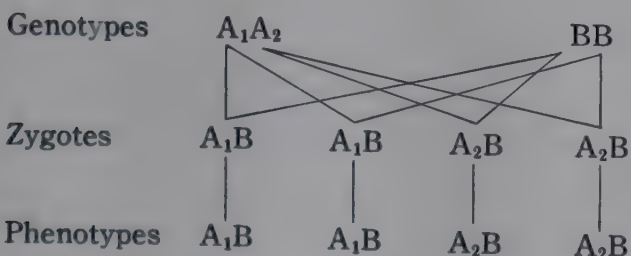
or



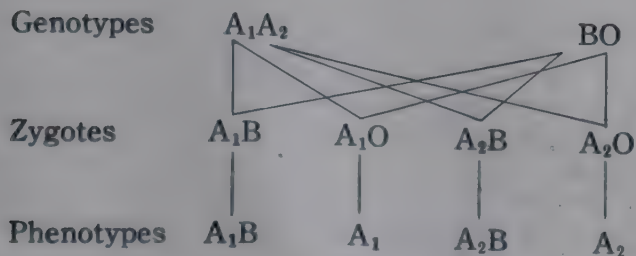
or



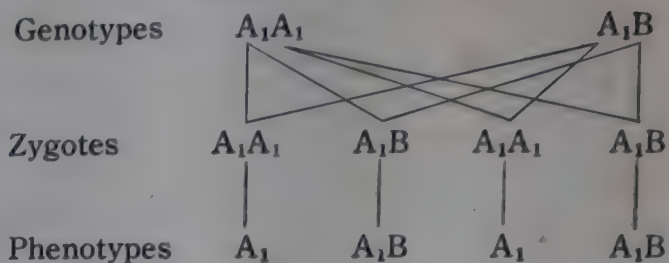
or



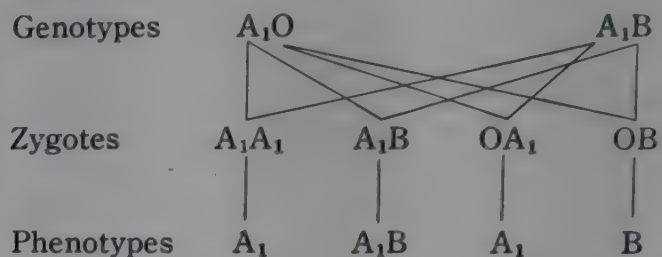
or

Children possible: O- A_1 - A_2 -B- A_1B - A_2B

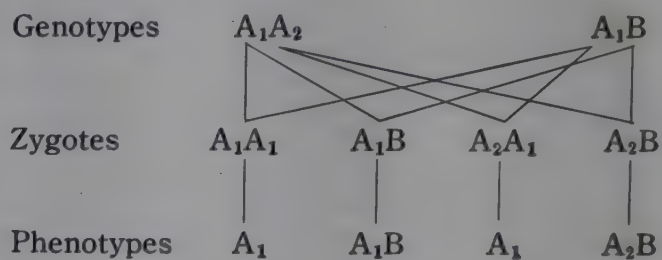
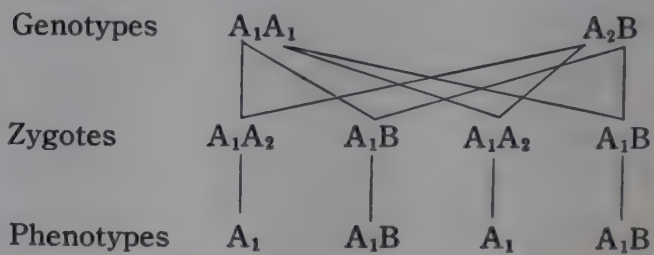
Children not possible: —

Parents: A_1 & A_1B 

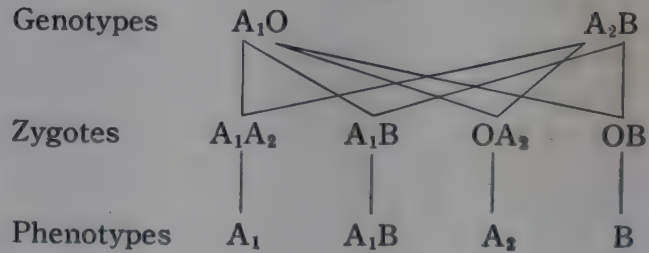
or



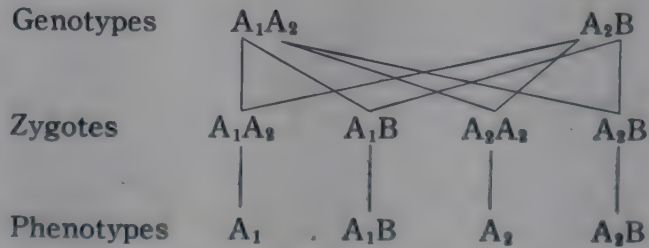
or

Children possible: A_1 -B- A_1B - A_2B Children not possible: O- A_2 Parents: A_1 & A_2B 

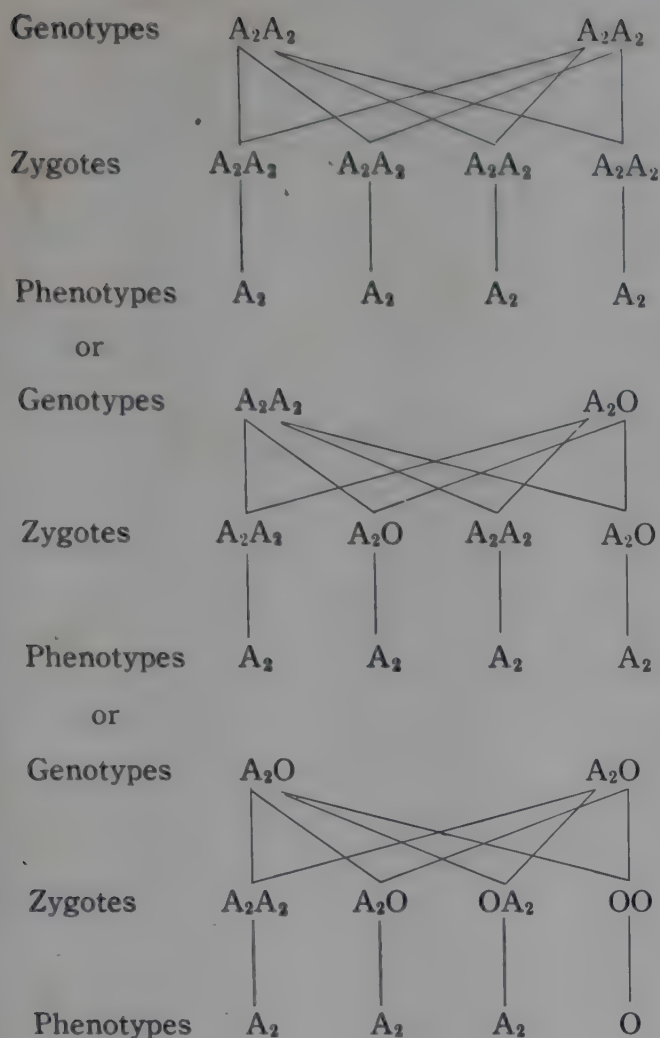
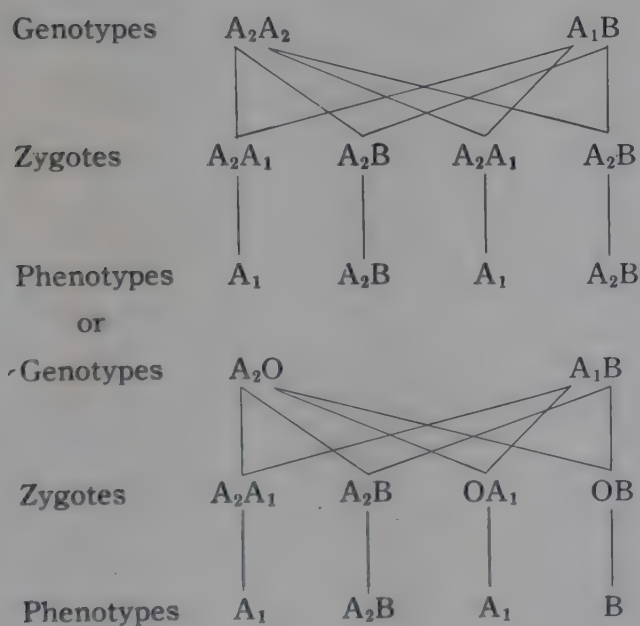
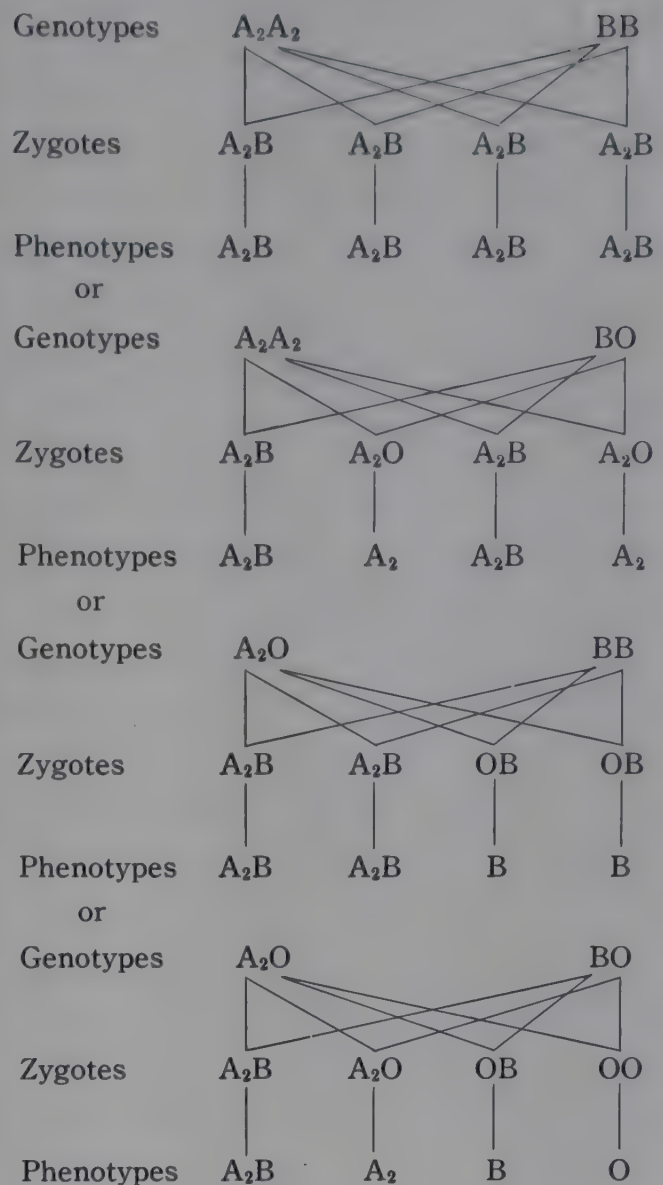
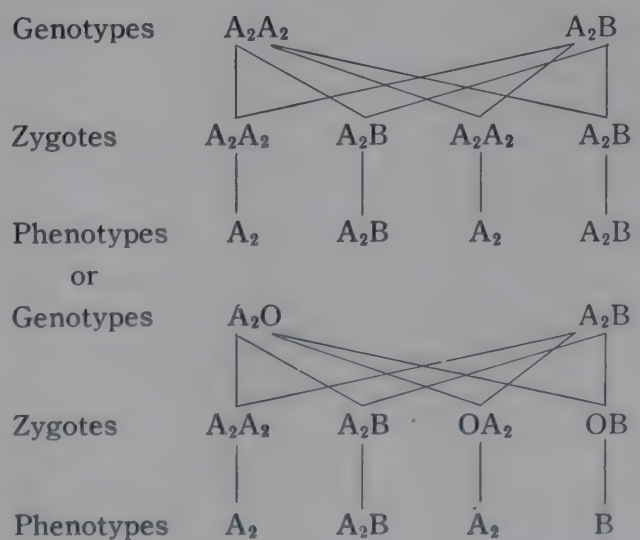
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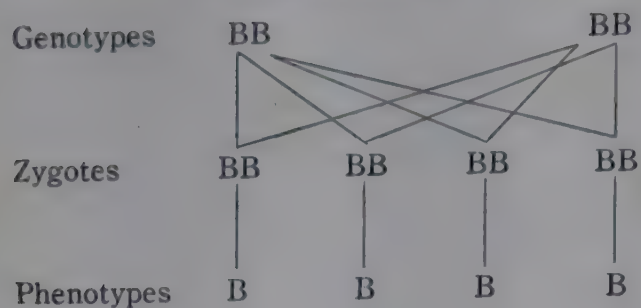


or

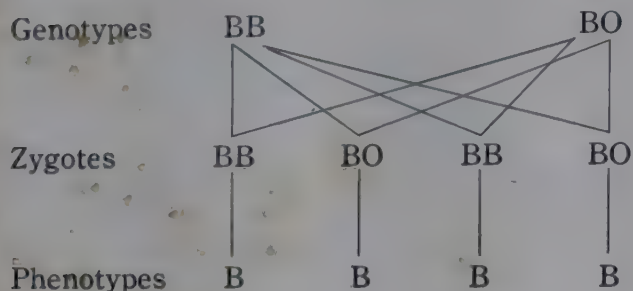
Children possible: A_1 - A_2 -B- A_1B - A_2B

Children not possible: O

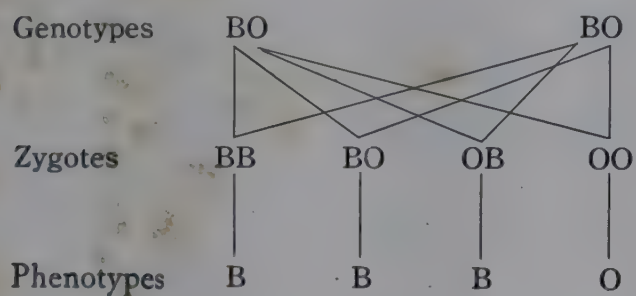
Parents: A_2 & A_2 Children possible: $O-A_2$ Children not possible: $A_1-B-A_1B-A_2B$ Parents: A_2 & A_1B Children possible: A_1-B-A_2B Children not possible: $O-A_2-A_1B$ Parents: A_2 & B Children possible: $O-A_2-B-A_2B$ Children not possible: A_1-A_1B Parents: A_2 & A_2B Children possible: A_2-B-A_2B Children not possible: $O-A_1-A_1B$

Parents: B & B

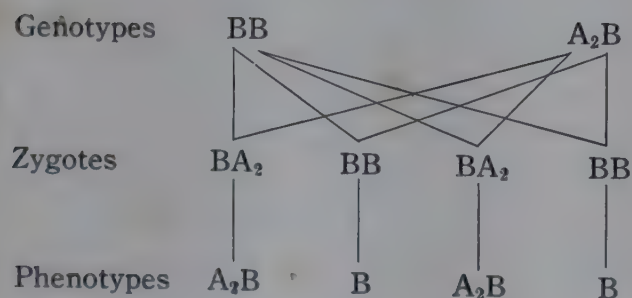
or



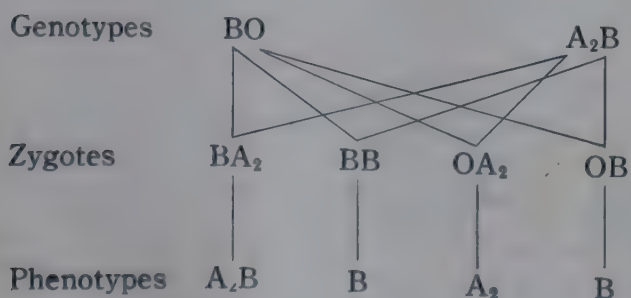
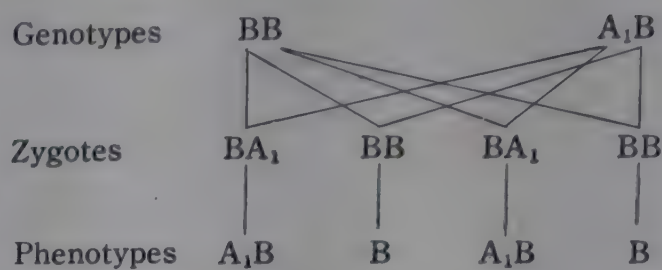
or



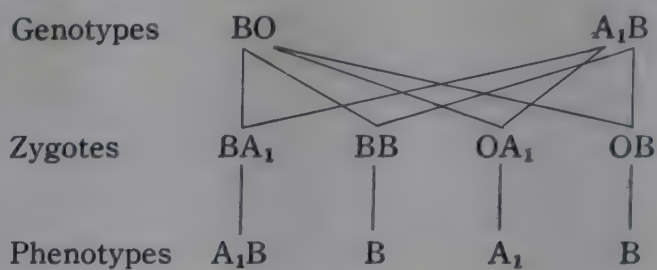
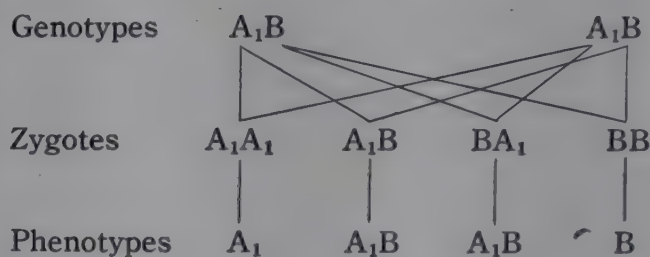
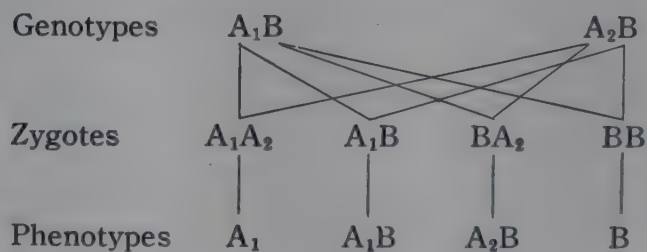
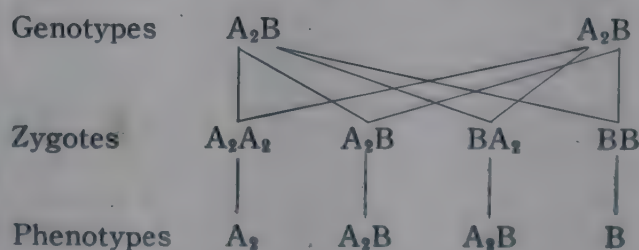
Children possible: O-B

Children not possible: $A_1-A_2-A_1B-A_2B$ Parents: B & A_2B 

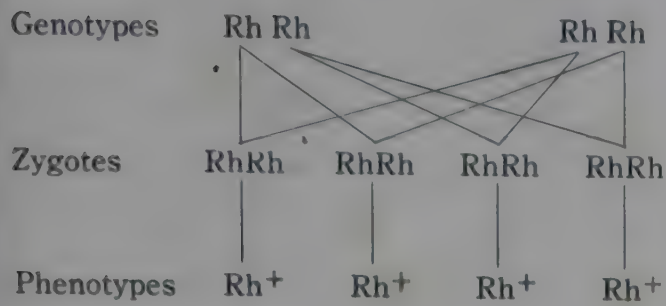
or

Children possible: A_2-B-A_2B Children not possible: $O-A_1-A_1B$ Parents: B & A_1B 

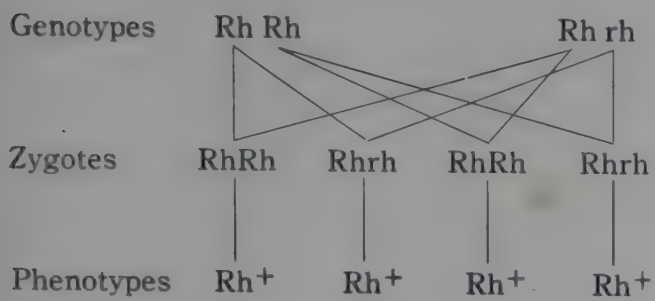
or

Children possible: A_1-B-A_1B Children not possible: $O-A_2-A_2B$ Parents: A_1B & A_1B Children possible: A_1-B-A_1B Children not possible: $O-A_2-A_2B$ Parents: A_1B & A_2B Children possible: $A_1-B-A_1B-A_2B$ Children not possible: $O-A_2$ Parents: A_2B & A_2B Children possible: A_2-B-A_2B Children not possible: $O-A_1-A_1B$

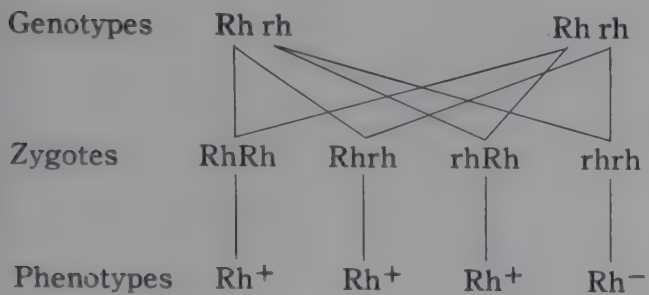
Parents: Rh⁺ & Rh⁺



or

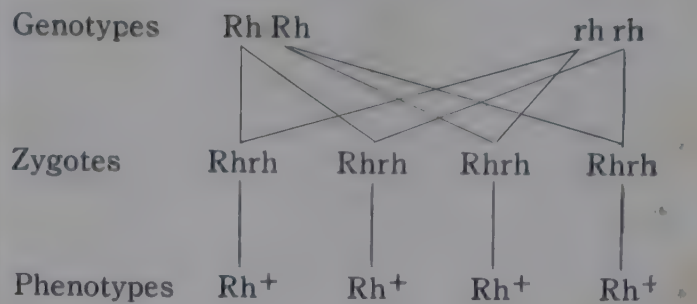


or

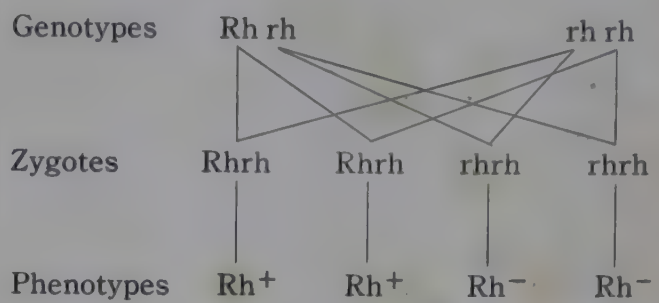


Children possible: Rh⁺ Rh⁻
Children not possible: —

Parents: Rh⁺ & Rh⁻

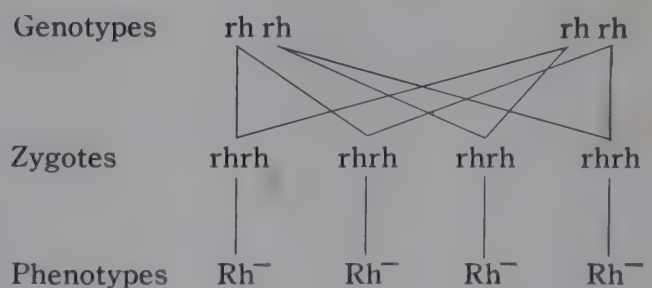


or



Children possible: Rh⁺ Rh⁻
Children not possible: —

Parents: Rh⁻ & Rh⁻



Children possible: Rh⁻
Children not possible: Rh⁺

TABLE 122.—SUMMARY OF HEREDITY FACTORS A, B, M, N, AND Rh*

PARENTS	POSSIBLE COMBINATIONS OF GENOTYPES	CHILDREN POSSIBLE	CHILDREN NOT POSSIBLE
M and M	MM and MM	M	MN, N
M and N	MM and NN	MN	N, M
M and MN	MM and MN	M, MN	N
N and N	NN and NN	N	M, MN
N and MN	NN and MN	N, MN	M
MN and MN	MN and MN	M, N, MN	—
O and O	OO and OO	O	A ₁ , A ₂ , B, A ₁ B, A ₂ B
O and A ₁	OO and A ₁ A ₁ OO and A ₁ A ₂ OO and A ₂ O	O, A ₁ , A ₂	B, A ₁ B, A ₂ B

(Continued on next page.)

*For a more complete discussion of the Rh factor refer to pages 1048, 1052 to 1054.

TABLE 122.—CONT'D

PARENTS	POSSIBLE COMBINATIONS OF GENOTYPES	CHILDREN POSSIBLE	CHILDREN NOT POSSIBLE
O and A ₂	$\begin{cases} \text{OO and A}_2\text{A}_2 \\ \text{OO and A}_2\text{O} \end{cases}$	O, A ₂	A ₁ , B, A ₁ B, A ₂ B
O and B	$\begin{cases} \text{OO and BB} \\ \text{OO and BO} \end{cases}$	O, B	A ₁ , A ₂ , A ₁ B, A ₂ B
O and A ₁ B	OO and A ₁ B	A ₁ , B	O, A ₂ , A ₁ B, A ₂ B
O and A ₂ B	OO and A ₂ B	A ₂ , B	O, A ₁ , A ₁ B, A ₂ B
A ₁ and A ₁	$\begin{cases} \text{A}_1\text{A}_1 \text{ and } \text{A}_1\text{A}_1 \\ \text{A}_1\text{A}_1 \text{ and } \text{A}_1\text{A}_2 \\ \text{A}_1\text{A}_1 \text{ and } \text{A}_1\text{O} \\ \text{A}_1\text{A}_2 \text{ and } \text{A}_1\text{A}_2 \\ \text{A}_1\text{A}_2 \text{ and } \text{A}_1\text{O} \\ \text{A}_1\text{O and } \text{A}_1\text{O} \end{cases}$	O, A ₁ , A ₂	B, A ₁ B, A ₂ B
A ₁ and A ₂	$\begin{cases} \text{A}_1\text{A}_1 \text{ and } \text{A}_2\text{A}_2 \\ \text{A}_1\text{A}_1 \text{ and } \text{A}_2\text{O} \\ \text{A}_1\text{A}_2 \text{ and } \text{A}_2\text{A}_2 \\ \text{A}_1\text{A}_2 \text{ and } \text{A}_2\text{O} \\ \text{A}_1\text{O and } \text{A}_2\text{A}_2 \\ \text{A}_1\text{O and } \text{A}_2\text{O} \end{cases}$	O, A ₁ , A ₂	B, A ₁ B, A ₂ B
A ₁ and B	$\begin{cases} \text{A}_1\text{A}_1 \text{ and } \text{BB} \\ \text{A}_1\text{A}_1 \text{ and } \text{BO} \\ \text{A}_1\text{A}_2 \text{ and } \text{BB} \\ \text{A}_1\text{A}_2 \text{ and } \text{BO} \\ \text{A}_1\text{O and } \text{BB} \\ \text{A}_1\text{O and } \text{BO} \end{cases}$	O, A ₁ , A ₂ , B, A ₁ B, A ₂ B	—
A ₁ and A ₁ B	$\begin{cases} \text{A}_1\text{A}_1 \text{ and } \text{A}_1\text{B} \\ \text{A}_1\text{A}_2 \text{ and } \text{A}_1\text{B} \\ \text{A}_1\text{O and } \text{A}_1\text{B} \end{cases}$	A ₁ , A ₁ B, A ₂ B, B	O, A ₂
A ₁ and A ₂ B	$\begin{cases} \text{A}_1\text{A}_1 \text{ and } \text{A}_2\text{B} \\ \text{A}_1\text{A}_2 \text{ and } \text{A}_2\text{B} \\ \text{A}_1\text{O and } \text{A}_2\text{B} \end{cases}$	A ₁ , A ₂ , B, A ₁ B, A ₂ B	O
A ₂ and A ₂	$\begin{cases} \text{A}_2\text{A}_2 \text{ and } \text{A}_2\text{A}_2 \\ \text{A}_2\text{A}_2 \text{ and } \text{A}_2\text{O} \\ \text{A}_2\text{O and } \text{A}_2\text{O} \end{cases}$	A ₂ , O	A ₁ , B, A ₁ B, A ₂ B
A ₂ and B	$\begin{cases} \text{A}_2\text{A}_2 \text{ and } \text{BB} \\ \text{A}_2\text{A}_2 \text{ and } \text{BO} \\ \text{A}_2\text{O and } \text{BB} \\ \text{A}_2\text{O and } \text{BO} \end{cases}$	O, A ₂ , B, A ₂ B	A ₁ , A ₁ B
A ₂ and A ₁ B	$\begin{cases} \text{A}_2\text{A}_2 \text{ and } \text{A}_1\text{B} \\ \text{A}_2\text{O and } \text{A}_1\text{B} \end{cases}$	A ₁ , B, A ₂ B	O, A ₂ , A ₁ B
A ₂ and A ₂ B	$\begin{cases} \text{A}_2\text{A}_2 \text{ and } \text{A}_2\text{B} \\ \text{A}_2\text{O and } \text{A}_2\text{B} \end{cases}$	A ₂ , B, A ₂ B	O, A ₁ , A ₁ B
B and B	$\begin{cases} \text{BB and BB} \\ \text{BB and BO} \\ \text{BO and BO} \end{cases}$	B, O	A ₁ , A ₂ , A ₁ B, A ₂ B
B and A ₁ B	$\begin{cases} \text{BB and A}_1\text{B} \\ \text{BO and A}_1\text{B} \end{cases}$	A ₁ , B, A ₁ B	O, A ₂ , A ₂ B
B and A ₂ B	$\begin{cases} \text{BB and A}_2\text{B} \\ \text{BO and A}_2\text{B} \end{cases}$	A ₂ , B, A ₂ B	O, A ₁ , A ₁ B
A ₁ B and A ₁ B	A ₁ B and A ₁ B	A ₁ , B, A ₁ B	O, A ₂ , A ₂ B
A ₁ B and A ₂ B	A ₁ B and A ₂ B	A ₁ , B, A ₁ B, A ₂ B	O, A ₂
A ₂ B and A ₂ B	A ₂ B and A ₂ B	A ₂ , B, A ₂ B	O, A ₁ , A ₁ B
Rh+ and Rh+	$\begin{cases} \text{RhRh and RhRh} \\ \text{RhRh and Rhrh} \\ \text{Rhrh and Rhrh} \end{cases}$	Rh+ and Rh-	—
Rh+ and Rh-	$\begin{cases} \text{RhRh and rhrh} \\ \text{Rhrh and rhrh} \end{cases}$	Rh+ and Rh-	—
Rh- and Rh-	rhrh and rhrh	Rh-	Rh+

TABLE 123*†

MATING	POSSIBLE COMBINATIONS OF GENOTYPES	CHILDREN POSSIBLE	CHILDREN NOT POSSIBLE
rh and rh	rr and rr	rh	rh' , rh'' , rh_y , Rh_0 , Rh_1 , Rh_2 , Rh_z
rh and rh'	rr and $r'r'$ rr and $r'r$	rh, rh'	rh'' , rh_y , Rh_0 , Rh_1 , Rh_2 , Rh_z
rh and rh''	rr and $r''r''$ rr and $r''r$	rh, rh''	rh' , rh_y , Rh_0 , Rh_1 , Rh_2 , Rh_z
rh and rh_y	rr and $r'r''^*$	rh' , rh''	rh, rh_y , Rh_0 , Rh_1 , Rh_2 , Rh_z
rh and Rh_0	rr and R^0R^0 rr and R^0r	rh, Rh_0	rh' , rh'' , rh_y , Rh_1 , Rh_2 , Rh_z
rh and Rh_1	rr and R^1R^1 rr and R^1r' rr and R^1r rr and R^1R^0 rr and $r'R^0$	rh, rh' , Rh_0 , Rh_1	rh'' , rh_y , Rh_2 , Rh_z
rh and Rh_2	rr and R^2R^2 rr and R^2r'' rr and R^2r rr and R^2R^0 rr and $r''R^0$	rh, rh'' , Rh_0 , Rh_2	rh' , rh_y , Rh_1 , Rh_z
rh and Rh_z ‡	rr and R^1R^2 rr and R^1r'' rr and $r'R^2$	rh' , rh'' , Rh_1 , Rh_2	rh, rh_y , Rh_0 , Rh_z
rh' and rh'	$r'r'$ and $r'r'$ $r'r'$ and $r'r$ $r'r$ and $r'r$	rh, rh'	rh'' , rh_y , Rh_0 , Rh_1 , Rh_2 , Rh_z
rh' and rh''	$r'r'$ and $r''r''$ $r'r'$ and $r''r$ $r'r$ and $r''r''$ $r'r$ and $r''r$	rh, rh' , rh'' , rh_y	Rh_0 , Rh_1 , Rh_2 , Rh_z
rh' and rh_y	$r'r'$ and $r'r''^*$ $r'r$ and $r'r''$	rh' , rh'' , rh_y	rh, Rh_0 , Rh_1 , Rh_2 , Rh_z
rh' and Rh_0	$r'r$ and R^0R^0 $r'r$ and R^0r $r'r$ and R^0R^0 $r'r$ and R^0r	rh, rh' , Rh_0 , Rh_1	rh'' , rh_y , Rh_2 , Rh_z

From Gradwohl: Legal Medicine, St. Louis, 1954, The C. V. Mosby Co.

*Tests for factors Rh_0 , rh' , and rh'' , only.†Does not include other rare possibilities involving gene r^y .‡Does not include genes R^2 and r^y .

TABLE 123—CONT'D

MATING	POSSIBLE COMBINATIONS OF GENOTYPES	CHILDREN POSSIBLE	CHILDREN NOT POSSIBLE
rh' and Rh ₁	$r'r'$ and R^1R^1 $r'r'$ and R^1r' $r'r'$ and R^1r $r'r'$ and R^1R^0 $r'r'$ and $r'R^0$ $r'r$ and R^1R^1 $r'r$ and R^1r' $r'r$ and R^1r $r'r$ and R^1R^0 $r'r$ and $r'R^0$	rh, rh', Rh ₀ , Rh ₁	rh'', rh _y , Rh ₂ , Rh _z
rh' and Rh ₂	$r'r'$ and R^2R^2 $r'r'$ and R^2r'' $r'r'$ and R^2r $r'r'$ and R^2R^0 $r'r'$ and $r''R^0$ $r'r$ and R^2R^2 $r'r$ and R^2r'' $r'r$ and R^2r $r'r$ and R^2R^0 $r'r$ and $r''R^0$	rh, rh', rh'', rh _y , Rh ₀ , Rh ₁ , Rh ₂ , Rh _z	
rh' and Rh _z	$r'r'$ and R^1R^2 $r'r'$ and R^1r'' $r'r'$ and $r'R^2$ $r'r$ and R^1R^2 $r'r$ and R^1r'' $r'r$ and $r'R^2$	rh', rh'', rh _y , Rh ₁ , Rh ₂ , Rh _z	rh, Rh ₀
rh'' and rh''	$r''r''$ and $r''r''$ $r''r''$ and $r''r$ $r''r$ and $r''r$	rh, rh''	rh', rh _y , Rh ₀ , Rh ₁ , Rh ₂ , Rh _z
rh'' and rh _y	$r''r''$ and $r'r''$ $r''r$ and $r'r''$	rh', rh'', rh _y	rh, Rh ₀ , Rh ₁ , Rh ₂ , Rh _z
rh'' and Rh ₀	$r''r''$ and R^0R^0 $r''r''$ and R^0r $r''r$ and R^0R^0 $r''r$ and R^0r	rh, rh'', Rh ₀ , Rh ₂	rh', rh _y , Rh ₁ , Rh _z
rh'' and Rh ₁	$r''r''$ and R^1R^1 $r''r''$ and R^1r' $r''r''$ and R^1r $r''r''$ and R^1R^0 $r''r''$ and $r'R^0$ $r''r$ and R^1R^1 $r''r$ and R^1r' $r''r$ and R^1r $r''r$ and R^1R^0 $r''r$ and $r'R^0$	rh, rh', rh'', rh _y , Rh ₀ , Rh ₁ , Rh ₂ , Rh _z	
rh'' and Rh ₂	$r''r''$ and R^2R^2 $r''r''$ and R^2r'' (Continued)	rh, rh'', Rh ₀ , Rh ₂	rh', rh _y , Rh ₁ , Rh _z

TABLE 123—CONT'D

MATING .	POSSIBLE COMBINATIONS OF GENOTYPES	CHILDREN POSSIBLE	CHILDREN NOT POSSIBLE
(cont'd)	$r''r''$ and R^2r $r''r''$ and R^2R^0 $r''r''$ and $r''R^0$ $r''r$ and R^2R^2 $r''r$ and R^2r'' $r''r$ and R^2r $r''r$ and R^2R^0 $r''r$ and $r''R^0$	rh, rh'', Rh_0, Rh_2	rh', rh_y, Rh_1, Rh_z
rh'' and Rh_z	$r''r''$ and R^1R^2 $r''r''$ and R^1r'' $r''r''$ and $r'R^2$ $r''r$ and R^1R^2 $r''r$ and R^1r'' $r''r$ and $r'R^2$	$rh', rh'', rh_y, Rh_1,$ Rh_2, Rh_z	rh, Rh_0
rh_y and rh_y	$r'r''$ and $r'r''$	rh', rh'', rh_y	$rh, Rh_0, Rh_1, Rh_2, Rh_z$
rh_y and Rh_0	$r'r''$ and R^0R^0 $r'r''$ and R^0r	rh', rh'', Rh_1, Rh_2	rh, rh_y, Rh_0, Rh_z
rh_y and Rh_1	$r'r''$ and R^1R^1 $r'r''$ and R^1r' $r'r''$ and R^1r $r'r''$ and R^1R^0 $r'r''$ and $r'R^0$	$rh', rh'', rh_y, Rh_1,$ Rh_2, Rh_z	rh, Rh_0
rh_y and Rh_2	$r'r''$ and R^2R^2 $r'r''$ and R^2r'' $r'r''$ and R^2r $r'r''$ and R^2R^0 $r'r''$ and $r''R^0$	$rh', rh'', rh_y, Rh_1,$ Rh_2, Rh_z	rh, Rh_0
rh_y and Rh_z	$r'r''$ and R^1R^2 $r'r''$ and R^1r'' $r'r''$ and $r'R^2$	$rh', rh'', rh_y, Rh_1,$ Rh_2, Rh_z	rh, Rh_0
Rh_0 and Rh_0	R^0R^0 and R^0R^0 R^0R^0 and R^0r R^0r and R^0r	rh, Rh_0	$rh', rh'', rh_y, Rh_1, Rh_2, Rh_z$
Rh_0 and Rh_1	R^0R^0 and R^1R^1 R^0R^0 and R^1r' R^0R^0 and R^1r R^0R^0 and R^1R^0 R^0R^0 and $r'R^0$ R^0r and R^1R^1 R^0r and R^1r' R^0r and R^1r R^0r and R^1R^0 R^0r and $r'R^0$	rh, rh', Rh_0, Rh_1	rh'', rh_y, Rh_2, Rh_z

TABLE 123—CONT'D

MATING	POSSIBLE COMBINATIONS OF GENOTYPES	CHILDREN POSSIBLE	CHILDREN NOT POSSIBLE
Rh_0 and Rh_2	R^0E^0 and R^2R^2 R^0E^0 and R^2r'' R^0E^0 and R^2r R^0E^0 and R^2R^0 R^0E^0 and $r''R^0$ R^0r and R^2R^2 R^0r and R^2r'' R^0r and R^2r R^0r and R^2R^0 R^0r and $r''R^0$	rh , rh'' , Rh_0 , Rh_2	rh' , rh_y , Rh_1 , Rh_z
Rh_0 and Rh_z	R^0E^0 and R^1R^2 R^0E^0 and R^1r'' R^0E^0 and R^2r' R^0r and R^1R^2 R^0r and R^1r'' R^0r and $r'R^2$	rh' , rh'' , Rh_1 , Rh_2	rh , rh_y , Rh_0 , Rh_z
Rh_1 and Rh_1	R^1R^1 and R^1R^1 R^1R^1 and R^1r' R^1R^1 and R^1r R^1R^1 and R^1R^0 R^1R^1 and $r'R^0$ R^1r' and R^1r' R^1r' and R^1r R^1r' and R^1R^0 R^1r' and $r'R^0$ R^1r and R^1r R^1r and R^1R^0 R^1r and $r'R^0$ R^1R^0 and R^1R^0 R^1R^0 and $r'R^0$ $r'R^0$ and $r'R^0$	rh , rh' , Rh_0 , Rh_1	rh'' , rh_y , Rh_2 , Rh_z
Rh_1 and Rh_2	R^1R^1 and R^2R^2 R^1R^1 and R^2r'' R^1R^1 and R^2r R^1R^1 and R^2R^0 R^1R^1 and $r''R^0$ R^1r' and R^2R^2 R^1r' and R^2r'' R^1r' and R^2r R^1r' and R^2R^0 R^1r' and $r''R^0$ R^1r and R^2R^2 R^1r and R^2r'' R^1r and R^2r R^1r and R^2R^0 R^1r and $r''R^0$ R^1R^0 and R^2R^2 R^1R^0 and R^2r'' R^1R^0 and R^2r R^1R^0 and R^2R^0 R^1R^0 and $r''R^0$ $r'R^0$ and R^2R^2 $r'R^0$ and R^2r'' $r'R^0$ and R^2r $r'R^0$ and R^2R^0 $r'R^0$ and $r''R^0$	rh , rh' , rh'' , rh_y , Rh_0 , Rh_1 , Rh_2 , Rh_z	

TABLE 123—CONT'D

MATING	POSSIBLE COMBINATIONS OF GENOTYPES	CHILDREN POSSIBLE	CHILDREN NOT POSSIBLE
Rh_1 and Rh_1	R^1R^1 and R^1R^2 R^1R^1 and R^1r'' R^1R^1 and $r'R^2$ R^1r' and R^1R^2 R^1r' and R^1r'' R^1r' and $r'R^2$ R^1r and R^1R^2 R^1r and R^1r'' R^1r and $r'R^2$ R^1R^0 and R^1R^2 R^1R^0 and R^1r'' R^1R^0 and $r'R^2$ $r'R^0$ and R^1R^2 $r'R^0$ and R^1r'' $r'R^0$ and $r'R^2$	rh' , rh'' , rh_y , Rh_1 , Rh_2 , Rh_z	rh , Rh_0
Rh_2 and Rh_2	R^2R^2 and R^2R^2 R^2R^2 and R^2r'' R^2R^2 and R^2r R^2R^2 and R^2R^0 R^2R^2 and $r''R^0$ R^2r'' and R^2r'' R^2r'' and R^2r R^2r'' and R^2R^0 R^2r'' and $r''R^0$ R^2r and R^2r R^2r and R^2R^0 R^2r and $r''R^0$ R^2R^0 and R^2R^0 R^2R^0 and $r''R^0$ $r''R^0$ and $r''R^0$	rh , rh'' , Rh_0 , Rh_2	rh' , rh_y , Rh_1 , Rh_z
Rh_2 and Rh_z	R^2R^2 and R^1R^2 R^2R^2 and R^1r'' R^2R^2 and $r'R^2$ R^2r'' and R^1R^2 R^2r'' and R^1r'' R^2r'' and $r'R^2$ R^2r and R^1R^2 R^2r and R^1r'' R^2r and $r'R^2$ R^2R^0 and R^1R^2 R^2R^0 and R^1r'' R^2R^0 and $r'R^2$ $r''R^0$ and R^1R^2 $r''R^0$ and R^1r'' $r''R^0$ and $r'R^2$	rh' , rh'' , rh_y , Rh_1 , Rh_2 , Rh_z	rh , Rh_0
Rh_z and Rh_z	R^1R^2 and R^1R^2 R^1R^2 and R^1r'' R^1R^2 and $r'R^2$ R^1r'' and R^1r'' R^1r'' and $r'R^2$ $r'R^2$ and $r'R^2$	rh' , rh'' , rh_y , Rh_1 , Rh_2 , Rh_z	rh , Rh_0

THE MEDICOLEGAL ASPECTS OF BLOOD GROUPING

It must be accepted that the specific agglutinating attribute of the red blood cells, the isoagglutinin, and the specific agglutinating power of the serum, the isoagglutinin, when once fully developed and established, persist in the blood of individuals without changing group throughout life, and this basic fact constitutes a test in a forensic way for the examination of blood stains and in disproving the possibility of paternity in certain disputed cases.

The use of this test for forensic purposes has certain limitations. We know that 87 per cent of the cases fall in groups O and A, and only 13 per cent in Groups B and AB. The tendency of the test may therefore more frequently suggest innocence rather than guilt, since the blood composing a stain on a suspect's clothing and that of the true murderer may belong to the same group. The true value of the test, according to Smith and Glaister¹ is apparent when the blood stains examined are of the same group as that of the victim but of a different group from that of the blood of the suspect; such a defense may be raised that the stains upon the accused's clothing were produced by his own blood. This grouping reaction narrows down the given number of individuals from whom a given sample of blood may have been derived.

Its use for the determination of paternity is of value, for although the true father can never be definitely identified by the test, it can frequently be asserted that a certain man is not the father of the child in question, or that he might be the father of the child. A man who fails in repeated blood grouping tests can definitely, and without doubt, be eliminated as the father. The test is also of value when a child is claimed by two pairs of parents.

I. THE IDENTIFICATION OF HUMAN BLOOD STAINS

Identification of blood stains as of human origin, including tests for the A-B-O and M factors, is of great value in cases of alleged hit-and-run driving, rape, murder, etc., or wherever stains suspected of being blood are found. Specimens are received in the laboratory on a variety of objects. These must all be carefully labeled in indelible ink, and the specimens must be kept for possible later presentation in court. This work must be done only by those properly authorized to perform the tests.

The author* has pointed out the importance of maintaining the integrity of chain of evidence. All evidence should be collected by members of the laboratory staff and by no one else, and the laboratory staff must work in close harmony with the homicide squad. Material should be brought to the Director's office, routinely entered in the laboratory book of entry, with time, names, etc., all written, and receipts properly given by those who receive the specimens. Full written data must be compiled covering who brought in the specimen, the time brought in, and the date. A receipt should be given to the member of the personnel who brings in the material and that member should also make a written statement that he has brought in the material. One must be prepared to submit all this written evidence during the trial of the case.

*Gradwohl, R. B. H.: *Lab. Digest* 18: No. 10, 1955.

¹Smith, S., and Glaister, J.: *Recent Advances in Forensic Medicine*, 1931, P. Blakiston's Son & Co.

Identifying a blood stain presents three problems. First, is the stain produced by blood? Second, if so, is this human blood? Third, to what blood group does it belong?

(a) Chemical Tests and Spectroscopic Examination

Chemical tests and spectroscopic examination will definitely establish the identity of a stain as blood or not blood.

1. The Benzidine Test.—

Make an extract of the material suspected of being blood. If this is in the form of scrapings, a very small amount of the scrapings may be used without dissolving, or it may be dissolved in physiologic saline. If the material is on cloth, cut the stained portion out and add a very small amount of saline to it. Use glassware which has never contained blood or blood products before. A control specimen must always be removed from an unstained portion, and tested along with the suspected stain. If the control portion gives a positive reaction, the test cannot be relied upon, and no further work may be done.

Make a saturated solution of benzidine (special for blood) in glacial acetic acid* by placing a small amount of the benzidine in a clean test tube, free from blood, and adding about 1 c.c. of glacial acetic acid to it. Shake vigorously. If all the crystals dissolve, add more benzidine until the acetic acid will not take up more of the solid. Add an equal volume of 3% hydrogen peroxide and mix thoroughly. If this solution turns green or blue, discard and make a fresh solution, using different glassware. The green or blue color was caused by the presence of blood in the glassware.

Place a drop of the extract of the suspected stain on a new clean glass slide and add a drop of the benzidine-glacial acetic acid mixture. A green or blue color indicates the presence of blood. Should this color fail to appear, repeat the test. If no color appears, the stain was not derived from blood and the test is terminated. If the specimen from the unstained portion of the material being examined gives a blue or green color, the test is without value and no further work may be done.

2. The Phenolphthalin Test.—See page 83.

3. Spectroscopic Examination of Blood Stains.—

Dissolve the stain in distilled water. The solution must be quite dilute.

Place the solution in a small glass chamber with parallel sides so arranged that rays of light will pass directly through it.

Place the chamber containing the dissolved stain in the spectroscope and adjust the instrument so that the spectrum is clearly visible. The blood will absorb some of the light rays from the spectrum, producing characteristic "absorption bands," which vary with the type of blood pigment present.

Oxyhemoglobin: 2 bands in the yellow between the Fraunhofer lines D and E.

Hemoglobin: A broad band between D and E, with a small rim over D on the red side.

Carboxyhemoglobin: Similar to oxyhemoglobin. Addition of ammonium sulphide reduces oxyhemoglobin to hemoglobin, but leaves carboxyhemoglobin unchanged.

Methemoglobin: A dark band in the red between C and D, but nearer C. If the solution is diluted properly, it splits into bands resembling acid hematin.

Acid hematin: A sharp band between C and D, nearer to C, and a broad band, not clearly defined, between D and F.

Alkaline hematin: A band between C and D. It also absorbs the violet end of the spectrum.

Hemochromogen: A sharp, dark band between D and E, and a pale, broader band over E.

Hematoporphyrin in alkaline solutions: A faint line between C and D, a broader band at D, and extending toward E, a band between D and E but nearly at E, and a broad band between E and F.

*The benzidine dehydrochloride test (page 84) may be used.

Old stains, when diluted, give hemoglobin and oxyhemoglobin bands. If a weak alkali is added, alkaline hematin is formed. If a weak acid is added, acid hematin is formed. Old and decomposed stains yield positive tests for hematoporphyrin. Add strong alkali to a portion of the blood solution to obtain alkaline hematoporphyrin bands.

Fresh blood shows oxyhemoglobin bands. Add a weak solution of ammonium sulphide to obtain the spectrum of hemoglobin. Add a dilute sodium hydroxide solution for hemochromogen bands. These two tests prove the presence of blood and also eliminate substances of vegetable and animal origin with spectra similar to that of oxyhemoglobin.

(b) Species Identification: The Precipitin Test

The principle of the precipitin test is based upon the work of Kraus who showed that, by immunizing a rabbit with injections of a culture of a bacterium, one could obtain a serum which, when added to a filtered culture of that bacterium, produces a precipitate in it.

Bordet¹ and Gengou² went further in carrying out this immunization experiment. Uhlenhuth worked out the same principle in relation to the precipitin test and its value as a medicolegal procedure. He injected rabbits with human blood and tested the antiserum on nineteen different bloods, finding a positive reaction only with human blood. Uhlenhuth³ published his tests on blood-stained articles such as sticks, sand, cotton, trousers, hatchet, etc. He showed that the antiserum made by injecting rabbits with human blood acted only with suspected human blood and that antiserum injected with pig's blood reacted only with pig's blood, etc. In other words, a precipitation occurs in the blood when in contact with homologous blood. Perhaps the most exhaustive of all these works was done by Nuttall⁴ who examined sera of all animals of any importance on the face of the globe.

Nuttall,⁴ in 1904, gave a classical account of blood relationships by means of the precipitin test. His work was done primarily to show that a common property exists in the blood of certain groups of animals, a property that has persisted throughout the ages which elapsed during our possible evolution from a common ancestor. He placed Man and Apes in one order, based on this work, the Anthropeidea.

Since the publication of Nuttall's book in 1904 and the present time, there have been occasional references in the literature on the specificity of the precipitin test.⁵ Gradwohl⁶ found that chimpanzee blood gave a ring reaction identical to that given by human blood, with antihuman precipitin serum, and that human blood gave an identical ring reaction with antichimpanzee precipitin serum. He further showed⁷ the same results using blood from the gorilla and the olive baboon (*Papio anubis*).

In other words, using the "ring" precipitin test, it is impossible to differentiate the bloods of these three primates and man. Duncan-Taylor and Gordon,⁸ by special alum absorption methods, believe that it is possible to

¹Bordet: Ann. Inst. Pasteur, 1899, p. 240.

²Gengou, O.: Ann. Inst. Pasteur, 1902, p. 734.

³Uhlenhuth, P.: Deutsche med. Wchnschr. 27: 1901.

⁴Nuttall, G.: Blood Immunity and Blood Relationship—The Precipitin Test for Blood. Cambridge, England, 1904, Cambridge University Press.

⁵Gradwohl, R. B. H.: Legal Medicine, St. Louis, 1954, The C. V. Mosby Co., p. 502 ff.

⁶Gradwohl, R. B. H.: Lab. Digest 15: Nos. 9, 12, 1952.

⁷Gradwohl, R. B. H.: Lab. Digest 17: No. 4, 1953.

⁸Duncan-Taylor and Gordon: See Gradwohl: Legal Medicine loc. cit.

produce a precipitin serum which will exclude the blood of primates when the "ring" test is made. Their method is given on page 1166. Gordon also examines blood stain extracts against an ox serum-absorbed antihuman precipitin serum, to establish that it is primate blood, but this absorbed serum is of a very poor potency.

There is no simple, practicable method available at present to distinguish between the blood stains of man and the primates (apes, monkeys including baboons, lemurs or nagapie).

There are two principal methods of carrying out the precipitin test: the ring test technic, and the flocculation test. The precipitins which are formed in animals by the injection of human blood are precipitins not for blood only, but for human protein. It is for this reason that this test can be used not only for the identification of blood but also for seminal stains, bone fragments, and meat adulteration.

It is very important that the antiserum which is to be used in the test be made properly and be tested on sufficient numbers of known positives to warrant its use in a given medicolegal procedure. These antisera react with groups of mammalian bloods and so in order to single out an individual of the mammalian group, one must resort to high dilutions. By high dilutions, is meant that the antiserum must be very potent and the material under investigation extremely well diluted for the actual test.

The use of a reliable antiserum is, of course, one of the most important considerations in making this test. Either prepare these antisera in the laboratory or purchase them from reliable biological houses. The latter method is probably better for those who do only an occasional precipitin test. Since these antisera are not likely to remain potent for a long time, only a small quantity should be purchased and that quantity should be kept in an efficient icebox. In preparation of the antisera, it is best to employ a large number of animals for these immunizations, at least six. Animals may be immunized by injecting human blood daily over a period of six weeks. After this time, a quantity of blood is taken from the ear and tested with a known positive human blood. If it is effective in a high dilution, the animals are bled to the point of exsanguination under aseptic precautions, and the blood collected in a sterile manner. Blood from these animals should be taken preferably several hours after the rabbits have eaten or during a period of fasting. If blood is taken from the animal immediately after a meal, the serum is likely to be opalescent; this is highly objectionable.

An antiserum to be efficient should so act that 0.1 c.c. when added to its respective serum-antigen in a dilution of 1 to 1,000 produces a turbidity either at once or in from one to five minutes at the latest, with the formation of a distinct precipitate in from thirty to sixty minutes.

Preparation of Antihuman Precipitin Serum

The following is the method used by the author. It gives excellent results.

Select six healthy rabbits. Make 50 c.c. of a 1:10 dilution of sterile human serum or plasma in saline, by adding to 5 c.c. of human serum or plasma (obtained under sterile conditions) 45 c.c. of sterile physiologic saline; mix.

Mark each rabbit with a tag or with indelible ink for identification, and keep complete records of each rabbit and each injection. Inject 2 c.c. of the 1:10 dilution of human serum

or plasma into the marginal ear vein of each rabbit. Beginning on Monday, and continuing through Saturday, make daily injections so that a total of six injections are given to each rabbit.

Allow one week to elapse, then inject each rabbit with 2 c.c. of the 1:10 dilution of human serum or plasma *intramuscularly* (to prevent anaphylaxis), followed by daily injections for the next five days, giving these five injections intravenously, as in the first week.

Allow one week's rest interval. Repeat the six injections of 2 c.c. of 1:10 dilution of human serum or plasma, giving the first injection intramuscularly and the next five intravenously.

Wait one week. Then withdraw a small quantity of blood from each rabbit and test to determine whether any antihuman precipitin substance has been developed. In other words, carry out an actual precipitin test as described below to determine whether the rabbit's serum will detect the presence of human blood. Usually one or more of the six rabbits will show this antihuman precipitating antibody. Estimate by dilutions the potency of this precipitating antibody. Reject all rabbits' sera not reacting in at least a 1:1000 dilution. Carry out controls with both positive and negative specimens.

If one or more of these rabbits has developed an antihuman precipitin of high titer, procure the entire content of blood from the selected rabbit or rabbits by dissecting the carotid artery and bleeding into sterile 50 c.c. Rockefeller centrifuge tubes, using all sterile precautions. (See page 2040 for method of bleeding rabbit from the carotid artery.)

Label the Rockefeller tubes carefully, and allow the blood to clot in a slanting position. Loosen the clots by striking the tubes against the palm of the hand, and centrifuge at high speed for at least twenty to thirty minutes to separate the serum. With all sterile precautions, carefully decant the supernatant serum, and place in sterile corked tubes. Inactivate at 56° C. to destroy the natural complement and to kill air bacteria which may have entered the blood during the procuring process. Add enough Merthiolate as a preservative to give a 1:10,000 concentration (0.1 c.c. of a 10% solution to 100 c.c. serum).

Ampule in 1 c.c. quantities in sterile glass ampules, using sterile precautions. Seal in the flame, label with rabbit number, date, and titer and store in the refrigerator.

Technic of the Precipitin "Ring" Test

Use 5 mm. tubing with a 2 mm. bore. This is very heavy-walled narrow glass tubing. Cut it into 12 cm. lengths and seal one end in the flame. These very small caliber test tubes are used in the precipitin test. They must never have contained human blood, and must be clear and dry.

Soak the material to be examined in saline for some time. When stains have been exposed to high temperatures, it is necessary to soak as long as seventy-two hours. This extraction should be carried out in a refrigerator to avoid bacterial action and turbidity. No chemical solvents except saline should be used, regardless of how difficult it may be to obtain an extract. If the extract contains debris or is cloudy, centrifuge and use the supernatant liquid for the test or filter through a Berkefeld filter. Use a minimal quantity of saline for the extraction.

Prepare a similar extract of the *unstained portion* of the material under examination. Make the dilution about the same as that of the stained area.

Various dilutions of the extract should be used, preferably a 1:100 and 1:1,000, although higher dilutions may be made and tested if desired. Dilutions of the stain of 1:1,000 or higher will show only a scanty foam upon shaking.

Prepare a dilution of *known human serum* of approximately 1:100 strength by adding 1 drop of human serum to 5 c.c. saline.

Prepare a *negative control serum* by adding one drop of rabbit blood serum to 5 c.c. saline (1:100).

Place antihuman precipitin serum in each of five small caliber tubes described above. Add this to a depth of about 5 cm. Label the tubes as follows: (1) blood stain; (2) unstained portion; (3) known human serum; (4) rabbit serum; (5) saline.

Stratify an *equal volume* of solution from the suspected stain on the serum in the first tube; use the extract from the unstained portion in the second tube; diluted human serum

in the third tube; diluted rabbit serum in the fourth tube; and saline in the fifth tube. This is accomplished by placing the solution above the serum so that it stands about a millimeter above it, then giving it a quick flick with the finger so that the two fluids come in contact with each other but do not mix.

Allow all tubes to stand at room temperature for five minutes. Read at the end of this time, but do not report definitely until 24 hours have elapsed. Keep the tubes in a refrigerator for the 24-hour period.

If the stain contains human blood, a white line will appear at the point of contact between the two liquids. This will be definite within five minutes. Confirm twenty-four hours later by examining for a plug of precipitate at the bottom of the positive tubes.

This result cannot be relied upon unless the control tests are as follows: negative reaction in tube 2, positive in tube 3, and negative in tubes 4 and 5. Positive reactions occur from human perspiration, urine, etc.

In carrying out the test, the numerous controls must be used, all carefully marked. If the object of the test is merely to identify the suspected stain as coming from a human source, the procedure can be rapidly carried out. But if the blood is not of human origin and an opinion is desired regarding its source, it then becomes necessary to test the blood serum in turn with different antisera until a positive result is obtained.

It has been found that human blood stains dried for ten to fifteen years may still show a positive reaction. Hansemann,¹ Meyer² and Friedenthal have given accounts of their examinations of mummy tissue with the use of precipitating antisera. The first obtained a positive reaction with such material 4,000 years old, the second with tissue 5,000 years old, while the last named failed to get a positive reaction with mummy tissue 500 years old. Nuttall³ has shown that if there is a mixture of several different bloods in solution, this does not prevent a reaction from taking place between an antiserum and its homologous blood in this test. This has been confirmed by Ziemke⁴ and Smith and Glaister.⁵ The effects of heat upon blood stains have been studied; it has been found that stains subjected to a temperature of 150° C. for fifteen minutes and 200° C. for two minutes still showed a positive precipitin test. It is to be emphasized, however, that blood that has been heated in this way requires a longer time to be soaked in order to produce an extraction.

Human blood stains that have been washed in water leaving only a faint color of the blood on the garment will still show the reaction.

Boiling blood stains does not necessarily cause the reaction to disappear. Smith and Glaister⁵ subjected material to the action of boiling water for from five minutes up to one hour and it still showed a precipitin reaction following extraction with saline for a period of twenty-four hours. Stains fourteen weeks old, however, similarly treated, failed to give a positive reaction on the application of a related antiserum. One must conclude, therefore, that in case of fresh stains, boiling quickly extracts the unfixed portion of the blood serum, but in older stains the degree of fixation does not readily permit the serum to pass into solution with the water, although prolonged steeping in saline thereafter successfully extracts a sufficient quantity to yield a positive reaction.

¹Hansemann: *Verhandl. d. physiol. Ges.*, Berlin, 1904.

²Meyer: *München. med. Wchnschr.*, p. 663, 1904.

³Nuttall: *Blood Immunity and Blood Relationship—The Precipitin Test for Blood*, 1904, p. 214.

⁴Ziemke: *Deutsche med. Wchnschr.* 27: 731, 1901.

⁵Smith, S., and Glaister, J., Jr.: *Recent Advances in Forensic Medicine*, Philadelphia, 1931. P. Blakiston's Son & Co.

In attempting to carry out this precipitin test on blood stains which have been soaked in soap powder, it has been found that the presence of soap powder contraindicates the use of this test for medicolegal purposes. Experiments on blood stains with various woods and leathers soaked with blood showed that the test is always reliable, provided the proper dilution is made.

Alum-Precipitated Precipitin Serum (Duncan-Taylor and Gordon)

Alum-Antigen.—

Dilute 25 c.c. of normal serum with 80 c.c. distilled water.

Add 90 c.c. of 10% potassium alum in distilled water.

Adjust the reaction to pH 6.5 with 5N sodium hydroxide solution, and centrifuge.

Wash the sediment twice with 200 c.c. of 1:10,000 Merthiolate-saline.

After centrifugalization, dilute the final precipitate to 100 c.c. with 1:10,000 Merthiolate-saline.

10 c.c. of the antigen are equivalent to 2.5 c.c. of serum.

Preparation of the Antiserum.—

Give 5 c.c. amounts intramuscularly into both hind legs of the rabbit.

Animals should weigh more than 4 kg. each and be in good condition.

Make experimental bleedings by a single puncture of the marginal ear vein on the twentieth day after injection. If sufficiently high titer is obtained, keep the animals without food for 24 hours and then bleed them from the carotid artery (page 2040), collecting the blood in sterile 50 c.c. Rockefeller tubes.

Allow the blood to clot in a slanting position, loosen the clot by striking the tube against the palm of the hand, centrifuge for about 30 minutes, and remove the serum, observing sterile precautions. Place in a sterile graduate.

Oxblood Absorption.—

Measure the serum.

Add 7% by volume ox serum to the antiserum.

Mix and maintain at room temperature for 2 hours, then place in a refrigerator at 5° C. overnight.

Centrifuge and remove the supernatant fluid.

Sterilize by filtration through a Seitz filter.

Ampule.

This alum-precipitated ox serum-absorbed antihuman precipitin serum will establish whether or not the blood stain is derived from primates, according to Gordon. When positive with ox-absorbed serum, the stain is not derived from primates.

If any of the primates is to be excluded by a precipitin test, the antihuman precipitin serum is prepared in an animal the species of which is to be excluded. Gradwohl* has reported results different from those of Taylor and Gordon, in that he obtained very little difference in the reaction of chimpanzee blood with absorbed or nonabsorbed antihuman precipitin serum. The reactions were almost indistinguishable.

Reliability of the "Ring" Test

1. A positive reaction means the presence of human blood.
2. Primate blood (chimpanzee, gorilla, olive baboon, lemur, etc.) will give an identical reaction.

*Loc. cit., Legal Medicine, p. 508.

3. Absorption of antihuman precipitin serum with oxblood does not necessarily remove the quality which gives a positive reaction with the antiserum and anthropoid blood.

The Flocculation Test for the Identification of Mammalian Bloods—The Libby Photronreflectometer (Photron'er)*

Gemeroy, Boyden, and DeFalco believe that by the use of a photronreflectometer to determine exact turbidities produced by mixing an antiserum with its corresponding antigen (antihuman precipitin serum with various dilutions of human sera), a serologic correspondence curve for that antiserum can be established, and if the serum is diluted sufficiently, it no longer reacts to a significant degree with any heterologous antigen tested against it in the native state; a different correspondence curve is established for this heterologous antigen. In other words, if an antihuman precipitin serum gives positive "ring" tests with both human and rhesus monkey bloods when the serum is not diluted, the probabilities are that it will not give powerful reactions with rhesus blood when the antiserum is diluted, although it will react strongly with human serum. In the preparation of an antiserum, it should therefore be possible to establish turbidity curves showing the type obtained with the antiserum in question and human serum, and the antiserum and other mammalian bloods. Gemeroy, Boyden, and DeFalco have conducted numerous experiments, and have produced normal titration curves of antihuman sera titrated with human, chimpanzee, and rhesus antigens. They report the serologic correspondence of the various antigens in percentage relationships with the curve produced by human serum (antigen).

To carry out this work, they use the Libby Photron'er,* which makes use of, or measures the amount of, scattered or reflected light that passes through a liquid. It is a delicate means of detecting finely suspended particles. A relatively small number of suspended particles reflects sufficient light to produce a significant reading on the galvanometer, and the greater the number of particles in suspension, the greater the amount of turbidity. A quantitative relationship can be established between the turbidity of a solution and the nitrogen content of a precipitate in protein antigen-antibody systems.

They use alum-precipitated serum for injection of rabbits. In the test, the antiserum is serially diluted with buffered saline.

Buffered Saline.—

Sodium Phosphate Solution: Dissolve 11.876 gm. of Na_2HPO_4 in distilled water and dilute to 1,000 c.c. with distilled water.

Potassium Phosphate Solution: Dissolve 9.078 gm. of KH_2PO_4 in distilled water and dilute to 1,000 c.c. with distilled water.

Add 6 c.c. of sodium phosphate solution

and 4 c.c. of potassium phosphate solution

to 50 c.c. of 0.85% sodium chloride solution, and dilute to 100 c.c. with 0.85% sodium chloride.

Serial dilutions of the serum, 1:250, 1:500, 1:1,000, 1:2,000, 1:4,000, 1:8,000, 1:16,000, 1:32,000, 1:64,000, 1:128,000, 1:256,000, and 1:512,000, are made with

*Gemeroy, A. B., and others: See Gradwohl: *Legal Medicine*, St. Louis, 1954, The C. V. Mosby Co., pp. 510 ff.

buffered saline. Each dilution is mixed with the antigen (serum being tested against the antiserum), and tested in the Photron'er. The sum of the turbidities noted from the complete reaction range of the antigen-antibody mixtures represents the precipitation capacity of the antiserum. Comparisons of total turbidities resulting from these summations represent the serologic correspondence of all the antigens tested with a single antiserum.

By making turbidity curves at various dilutions, using the antiserum against different bloods, one can determine the exact dilution of the antiserum where further dilution would practically "wipe out" any reaction with certain bloods, thereby making this reaction almost species specific. It is possible that this flocculation test may find wider use in identifying specific blood when more facts have been established. For complete details, refer to *Legal Medicine* (1.c.).

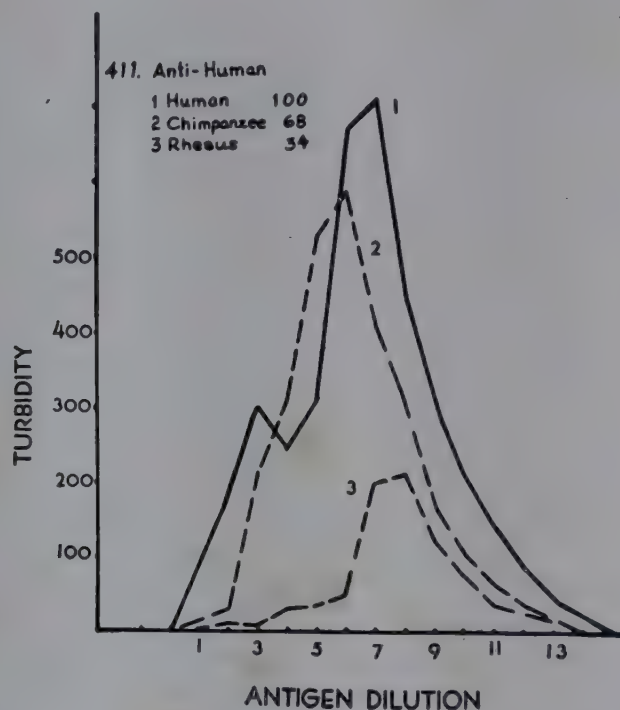


Fig. 287.—Normal titration curves when an antihuman serum is titrated with human, chimpanzee, and rhesus antigens. The serologic correspondence of these antigens is recorded in percentage relationships at the top of the figure. (From Gradwohl: *Legal Medicine*; The C. V. Mosby Co.)

(c) Blood Grouping Tests

The third problem to be solved is the determination of the group of blood to which this human blood stain belongs. For practical purposes, the investigation must be limited to a search for the A, B, O, and AB groups, and at times for the M factor. Results of attempted identification of blood stains for the Rh types are rather disappointing. The N factor disappears rapidly in dried blood, but the M factor can be found at times, so that tests for the M-N types are likewise disappointing.

Identification of the various blood factors in a dried stain is often used in forensic medicine to prove that a certain stain did *not* come from a certain individual. If, in a given case, the blood group of the suspect and that of the victim are the same, no deductions can be drawn, for the stain might have come from the suspect. One must remember that a stain on clothing may be

due to group receptors coming from the individual himself. Underclothing worn for several days is almost certain to contain A and B receptors if these are present in the blood and sweat.

But if the investigator obtains different results with the stain found on the suspect and the suspect's own blood, then evidently the stain was derived from a different individual. And if the stain has the same blood factors as the blood of the victim, it might or might not have been derived from the victim.

If liquid blood is submitted, tests are run in the same manner as those used in blood grouping for transfusions. If the blood is submitted in the form of a "crust," and this blood is fairly fresh (not more than 48 hours old), and sufficient material is submitted, the crust test may be run. Usually, however, one resorts to the agglutinin absorption test to identify blood stains.

Examination of Blood Crusts.—

Place small portions of the stain of approximately equal weight on glass slides.

Add, to one, a suspension of group A₁ cells in saline, and to the other a suspension of group B cells.

After the crust has softened, drop cover slips over the mixtures.

Apply gentle pressure intermittently to the cover slip and examine from time to time under the low power of a microscope, for a time period of about 30 minutes.

At the same time, add a suspension of group O cells to some of the crust, and treat in the same manner as for A and B cells. This is the control. These group O cells must not be agglutinated. If they are, the results of the tests are not reliable.

If A cells are agglutinated, the stain contains anti-A agglutinin; if the B cells are agglutinated, the stain contains anti-B.

TABLE 124.—POSSIBLE RESULTS WITH THE CRUST TEST

BLOOD CRUST PLUS A ₁ CELLS	BLOOD CRUST PLUS B CELLS	AGGLUTININ PRESENT	CONCLUSIONS
+	+	Anti-A Anti-B	Blood stain derived from group O individual
+	—	Anti-A	Blood derived from group B, or else failure of reaction with B cells
—	+	Anti-B	Blood derived from group A, or else failure of reaction with A cells
—	—	None	Either stain is group AB or there is lack of reaction due to disappearance of agglutinins

Agglutinin Absorption Test.*—

The agglutinin absorption test is made to determine the agglutinin content of a blood stain. When the stain is mixed with anti-A, anti-B, or anti-M serum, removal of the agglutinin present in the antiserum means the presence in the stain of the corresponding agglutinin. In other words, agglutinin "A" will absorb agglutinin anti-A, agglutinin "B" will absorb anti-B, etc. Anti-A serum normally agglutinates cells containing the agglutinin "A"; therefore, if these cells are added to the antiserum which has been subjected to absorption by the stain, and the "A" cells do not agglutinate, the stain has removed the anti-A agglutinin from the antiserum. This proves that the stain contains "A" agglutinin. This principle is true for anti-B and anti-M sera.

*These tests can be made on gastric contents, semen, etc.

Since the cells themselves cannot be recovered from a dried blood stain, no actual blood grouping experiment can be made, and resort must be had, therefore, to the agglutinin absorption method.

If the blood is in the form of a crust or powder, and sufficient specimen is obtainable, grind it into very fine powder, and add 10 mg. portions to 0.1 c.c. of each testing serum (anti-**A**, anti-**B**, and anti-**M**) in small tubes.

Mix thoroughly with the aid of a stirring rod and then allow it to stand in a refrigerator overnight to complete the absorption. Centrifuge and test the supernatant dark-red fluid for its agglutinin content (see above).

If the material has been absorbed, as it will be on clothing, paper, and other articles, cut out portions of the stained and also the unstained areas, of approximately equal size, and use these for the tests. It is essential that controls be run, using material from the unstained portion of material, since this at times contains one or both of the blood group receptors, from sweat, urine, or animal material. If the tests for the receptors in the unstained material are positive, the results obtained in tests on the stained area cannot be relied upon.

Use glassware and equipment which contains no blood or body secretions. Never touch any of the material with the bare hand. Always mark the portion of specimen from which the stain and the unstained areas have been cut, using an indelible pencil, for possible later identification in court.

Material Needed.—

Have on hand anti-**A**, anti-**B**, and anti-**M** testing sera with titers not to exceed 1:8 or 1:16. If the titers are higher than this, make appropriate dilutions.

There must also be saline suspensions of group **A**, **B**, and **OM** cells.

Technic.—

Cut out portions of both stained and unstained material of approximately equal size, and place in separate small Kahn tubes. Use three tubes for the stained portion and three for the unstained portion.

To each tube 1, add anti-**A** serum, only as much as the specimen will absorb after prodding with a narrow stirring rod.

Add anti-**B** serum to each tube 2, and anti-**M** serum to each tube 3.

Place in a refrigerator overnight. Enough serum can be recovered for the tests by pressing the tip of a capillary dropper against the material and sucking up with a rubber bulb. Tests and controls are run simultaneously, as in Table 125.

TABLE 125

TUBE 1	2	3	4	5	6
Anti- A serum plus Stain	Anti- A serum plus Unstained	Anti- B serum plus Stain	Anti- B serum plus Unstained	Anti- M serum plus Stain	Anti- M serum plus Unstained

Place in a refrigerator overnight.

The following day, express serum as directed above, using a capillary dropper, and test against cells as directed in Table 126. Carry out the tests on ring slides (Boerner).

Add the opposing cells to the serum subjected to absorption, rotate for 5 to 10 minutes, and examine under the low power of a microscope. Report agglutination as positive and lack of agglutination as negative.

Rings 2, 8, and 10 must show agglutination: Anti-**A** serum plus unstained material, plus **A** cells; anti-**B** serum plus unstained material, plus **B** cells; and anti-**M** serum plus unstained material plus **M** cells. These controls must always be positive.

Rings 3, 4, 5, and 6 must always be negative: anti-**B** serum plus **A** cells, anti-**A** serum plus **B** cells.

Rings 1, 7, and 9 are the test rings, the others being controls. See Table 127.

TABLE 126

1	2	3	4
Extract from tube 1	Extract from tube 2	Extract from tube 3	Extract from tube 4
⁺	⁺	⁺	⁺
A cells	A cells	A cells	A cells
5	6	7	8
Extract from tube 1	Extract from tube 2	Extract from tube 3	Extract from tube 4
⁺	⁺	⁺	⁺
B cells	B cells	B cells	B cells
9	10		
Extract from tube 5	Extract from tube 6		
⁺	⁺		
M cells	M cells		

TABLE 127.—RESULTS POSSIBLE IN THE TEST

RING 1	RING 7	RING 9	
Extract from	Extract from	Extract from	
tube 1	tube 3	tube 5	
⁺	⁺	⁺	
A cells	B cells	M cells	CONCLUSIONS
-	-		Stain contained both A and B agglu- tinogens, which removed anti-A from tube 1 and anti-B from tube 3: Stain belongs to group AB*
-	+		Stain removed anti-A from tube 1 and therefore contained "A" agglu- tinogen. It did not remove anti-B from tube 3 and therefore did not contain "B" agglutininogen. Stain is group A (or it might have failed to absorb the anti-B, in which case it would be AB). Stain contains the "A" agglutininogen
+	-		Stain did not remove anti-A from tube 1 but did remove anti-B from tube 3. The stain contained only "B" agglutininogen, or else failed to ab- sorb the anti-A. In the first case, it is derived from group B indi- vidual. It contains "B" agglu- tinogen
+	+		Stain removed neither anti-A nor anti- B and therefore contained no ag- glutininogen, group O, or else there was failure of absorption
		-	Stain removed anti-M and therefore contained M agglutininogen
		+	Stain failed to remove anti-M and thus either does not contain M agglutininogen or else there was failure of absorption

*This is always a dangerous diagnosis to make because of the possibility of non-specific absorption.

One must remember that these are methods for detecting indirectly the presence of certain agglutinogens in a blood stain, and are not direct tests for blood groups as entities. One may state that a stain contained a certain agglutininogen, but not that a certain agglutininogen was not present.

BLOOD GROUPING TESTS IN THE EXCLUSION OF PATERNITY

Since the agglutinogens O, A, B, M, N, Rh, and Hr are inherited, blood grouping can be applied to the problem of paternity. There are certain fundamental facts on which this statement is made. First, agglutinogens A and B or any others cannot appear in the blood of a child unless present in the blood of one or both parents. Second, an AB parent cannot give rise to a group O child, nor can an O parent give rise to an AB child. According to our present knowledge, there are 288 different combinations of blood groups, so the problem of determination of parentage by blood grouping has been greatly elaborated. Both cells and serum of each individual should be examined, together with the usual control tests. In Tables 122 and 123 will be found a summary of the hereditary facts of A, B, M, N, and Rh, with the possibility of blood groups in children of all the matings and also the offspring not possible.

While it is true that isoagglutinins are not fully developed until the child is several months old, the blood group can be determined from the agglutinogens in all cases. Wiener gives the following examples of cases in which blood grouping may prove of value: (1) in cases of children born out of lawful wedlock where the husbands deny paternity; (2) in cases where children are born out of lawful wedlock and the men named by the mothers as their fathers deny paternity; (3) in cases where newborn infants have been accidentally interchanged in the hospital and it is necessary to identify the parents of the infants; or where a wet nurse has willfully substituted her own infant for the one placed in her custody; (4) in cases where women have simulated pregnancy and childbirth in order to compel men to marry them or to obtain dower rights in the deceased husbands' estates.

Blood grouping is of value only for excluding paternity. With our present knowledge paternity cannot be definitely proved. It may be difficult or even impossible at times to establish the innocence of a putative father falsely accused. For example, if both the mother and the infant are group O MN and the putative father is group OMN, no conclusions can be drawn except that the accused could be the father of the child. If, however, he is group AB, he is at once exonerated, since no AB individual can be the parent of a group O child.

It is to be borne in mind that there are many different combinations or kinds of blood obtainable. Wiener¹ has shown by utilizing the four major blood groups, O, B, A, and AB, with subgroups of A and AB; the three blood types M, N, and MN; and the eight Rh blood types, it is now possible to distinguish 144 different kinds of human blood instead of the original four groups. There are six groups, O, A₁, A₂, B, A₁B and A₂B. There are three blood types, M, N, and MN, and two types due to the absence or presence of factor P. By multiplying together the number of subgroups of each of the above, as well as the eight Rh types, namely, $6 \times 3 \times 8$, the figure 144 is obtained. If the **hr'** and **hr''** factors are also utilized, the number is even greater. Only a few contributions

¹Wiener, A. S.: *Blood Groups in Blood Transfusion*, ed. 3, Springfield, Ill., 1943, Charles C Thomas, Publisher.

on the subject simultaneously utilizing the three blood group systems have appeared in the literature, namely, by Wiener,² by Alvarez,³ and the most recent by Unger.⁴ (Note: The **P** factor is not used in paternity cases.)

The following cases in our service* illustrate the manner of using blood grouping tests for A-B and M-N factors in cases of disputed paternity.

Case 1. This was a case in which the husband feared that he was not the father of his 4-year-old child, although he stated that he had no reason to doubt the faithfulness of his wife. The father wanted this test carried out because one of his friends, evidently a trouble maker, had suggested to him in a bantering manner that he might not be the father of his child. This caused him such mental unrest that he determined to have the tests made to ease his mind. Fortunately, we were able to accomplish this purpose.

The mother, child, and husband were tested in the usual manner for blood groups A-B, and Types M-N. The results of the tests were as follows:

Mother was OMN

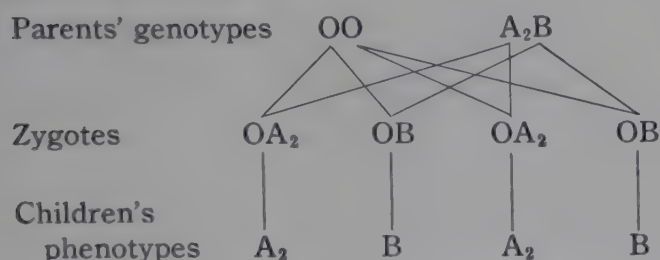
Child was A₂N

Father was A₂BN

With this rather rare combination of blood groups, it was very easy to state that this man could have been the father of the child, although, as stated above, actual paternity cannot be definitely proved by these tests. Since the child possessed the agglutinin N, which was present also in the mother's blood, the father must necessarily have had agglutinin N in his erythrocytes. With a group O mother and a group A₂B father, the children will be either A₂ or B, but not O or A₂B. (See Table 122.)

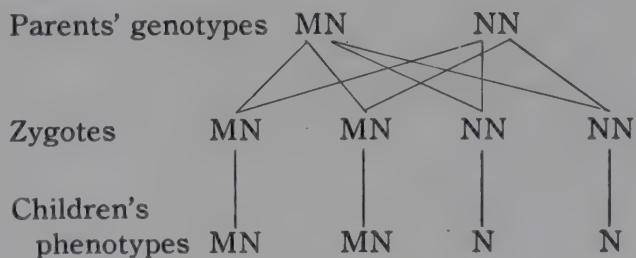
Genetics for this case would be expressed as follows:

Mating O and A₂B



Children possible: — A₂-B-MN-N

Mating MN and N



Children not possible: — O-A₁-A₁B-A₂B-M

Case 2.—This was a case of an unmarried mother, whose blood group was OM. The putative father denied the charges of paternity but was willing to submit to having tests made of his blood. The baby in this case was group OMN. Tests of the man's blood showed him to be Group OM. In this instance, the factors A-B were not conclusive evidence of paternity, but the types M-N showed definitely that the man in question could not be the father of the child. He was, therefore, relieved of the responsibility of supporting a child which was not his own. If the mother is type M, and the child is type MN, the father must be either type N or MN (consult Table 122), for he must have the N agglutinin in his erythrocytes.

Had the man in this case been Type N or MN, then the laboratory tests would have proved nothing beyond the fact that he could have been the father of the child.

*Gradwohl Laboratories, St. Louis, Mo.

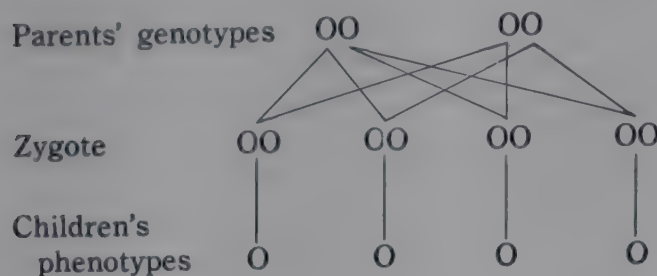
²Wiener and Sonn: J. Lab. & Clin. Med. 30: 395, 1945. Wiener: Am. J. Clin. Path. 16: 477, 1946.

³Alvarez: Bol. Asoc. Med. Santiago 6: 463, 1948.

⁴Unger: J.A.M.A. 152: 1006, 1953.

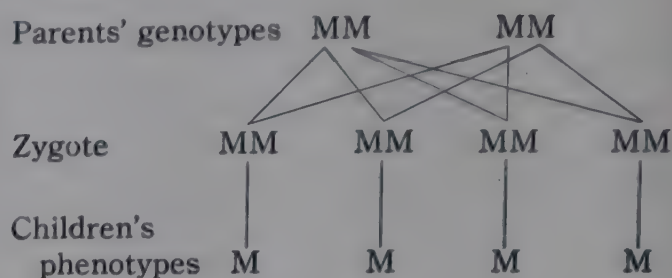
Genetics for this case are as follows:

Mating O and O



Children possible: — O — M

Mating M and M



Children not possible: — A₁-A₂-B-A₁B-A₂B-N-MN

According to the work of Landsteiner and Wiener,¹ it has been shown that the property Rh is not a sex-linked dominant factor. This follows first from the equal distribution of the factor in the two sexes, and second, from the analysis of families in which the father is positive and the mother is negative, where on the usual hypothesis only the offspring of one sex, most probably the daughters, would exhibit the character.

Facts Concerning the Heredity of Blood Groups.—

The M factor cannot appear in a child unless it is present in the parent; similarly, the N factor must be present in a parent in order to appear in a child.

If a child is an M type, the M factor must have been present in both parents. If the child is type N, the N factor was present in both parents.

If one or both parents are M, the offspring cannot be type N.

If either or both parents are N, the children cannot be type M.

An AB individual cannot be the parent of an O child.

An O individual cannot be the parent of an AB child.

The erythrocytic structures A and B are found in children only if present in the parents.

If this structure is absent in the parents, it is never present in the children.

If both parents have a definite blood structure, it usually appears in all the children; however, it may be absent in some.

If only father or mother possesses a definite structure, it is usually found in some of the children, but it may be found in all.

For instance, if a child has A erythrocytes, but the mother has no A factor in her cells, then the father must have A erythrocytes. If the father has no A erythrocytes, but he has O or B corpuscles, his parenthood is disproved.

From a medical standpoint we have been long interested in the problem of possible exceptions to this rule. A Prussian court stated: "if 2,000 children were examined and 1 exception would be found, this court would doubt the reliability of blood typing." The obstacles to the legal recognition of blood typing are removed. On the basis of blood findings of mother and child and the presumed father, it has been proved that it is apparently impossible

¹Landsteiner, K., and Wiener, A. S.: J. Exper. Med. 74: 309, 1941.

that X is the father of the child, or else the fatherhood of X is possible but cannot be proved positively. Another man of the same blood group could be the father. A diagnosis based on blood groups may, therefore, lead to the exclusion of fatherhood but it cannot yield results in a positive sense. If, in addition to A and B, we take blood properties M or N into consideration, according to Schiff, we can recognize serologically every third "non-father."

When the A-B-O, M-N, and Rh-Hr blood group systems are simultaneously utilized, Unger² states that there is an approximate 50 per cent chance of excluding a falsely accused man of being the father of a given child.

Technic of Blood Grouping Tests for the Exclusion of Paternity

All tests are made in duplicate, using two sets of antisera from two different manufacturing sources. If these are not available, then use two sets of antisera from the same manufacturer, but from different batches of sera. All tests must be read by two different people, without knowledge of the results obtained by one another (the "blind" test of Wiener).

It is necessary to have blood specimens from (a) the mother, (b) the baby, and (c) the putative father.

No Rh-Hr sensitivity tests need be made on these bloods.

Clotted blood is used for obtaining serum. Loosen the clot, centrifuge, and remove the supernatant serum, placing it in a properly labeled tube. Do this for all three bloods.

Liquid blood is obtained by taking the blood in the special dry anticoagulant (page 1005) and shaking to prevent clotting. The supernatant plasma may be used if no clotted blood has been submitted, but results are more clear-cut with serum than with plasma.

If only clotted blood is submitted, the 2 per cent cell suspension in saline and the 50 per cent cell suspension in their own plasma or serum must be made from the clot after the serum has been removed. Shake some of the cells loose from the clot in the serum, and centrifuge. Remove enough serum to leave a 50 per cent concentration of cells. Prepare the 2 per cent saline suspension from the clot by shaking the cells free into saline.

If blood is taken in an anticoagulant, prepare the cell suspensions as outlined on page 1005.

Wash the saline-suspended cells once with saline, and resuspend to a 2 per cent concentration in saline.

The following antisera will be needed:

Anti-A	Anti-Rh ₀ saline agglutinating
Anti-B	Anti-Rh ₀ slide test (blocking)
Absorbed anti-A	anti-hr' saline agglutinating*
Anti-M	anti-hr'' saline agglutinating (if
Anti-N	available)*
Anti-rh' saline agglutinating	anti-hr' slide test (blocking)*
Anti-rh'' saline agglutinating	anti-hr'' slide test (blocking)*

*Either the saline agglutinating serum or the slide test serum may be used. It is not necessary to use both.

²Unger, L. J.: J.A.M.A. 152: 1006, 1953.

The following tests are made of all the blood specimens: mother's, father's, and baby's. (See Fig. 288, *A* and *B*.)

1. Test the cells for A-B-O groups by mixing the cells with anti-**A** and also anti-**B** sera of high potency. At the same time, make controls on the antisera. Exact technic is given on page 1012.

2. Check the results in (1) by testing the serum or plasma against known A and B cells suspended in saline. If different results are obtained in these two tests, an error has been made and the tests must be repeated, using different antisera. If isohemolysis occurs, and the results cannot be easily read, inactivate the serum, cool to room temperature, and repeat the test.



Fig. 288A.—Setup for blood-grouping tests in determination of paternity.

3. Subgroup all A's and AB's by using the cell suspensions against absorbed anti-**A** serum (anti-**A**₁) on which proper controls have been made. See page 1018.

4. Test the cells against anti-**M** and anti-**N** sera on which controls have been run. This gives the M-N types (see page 1144).

5. Make tube agglutinating tests for the **Rh** and **Hr** factors and the **Rh**₀ variant:

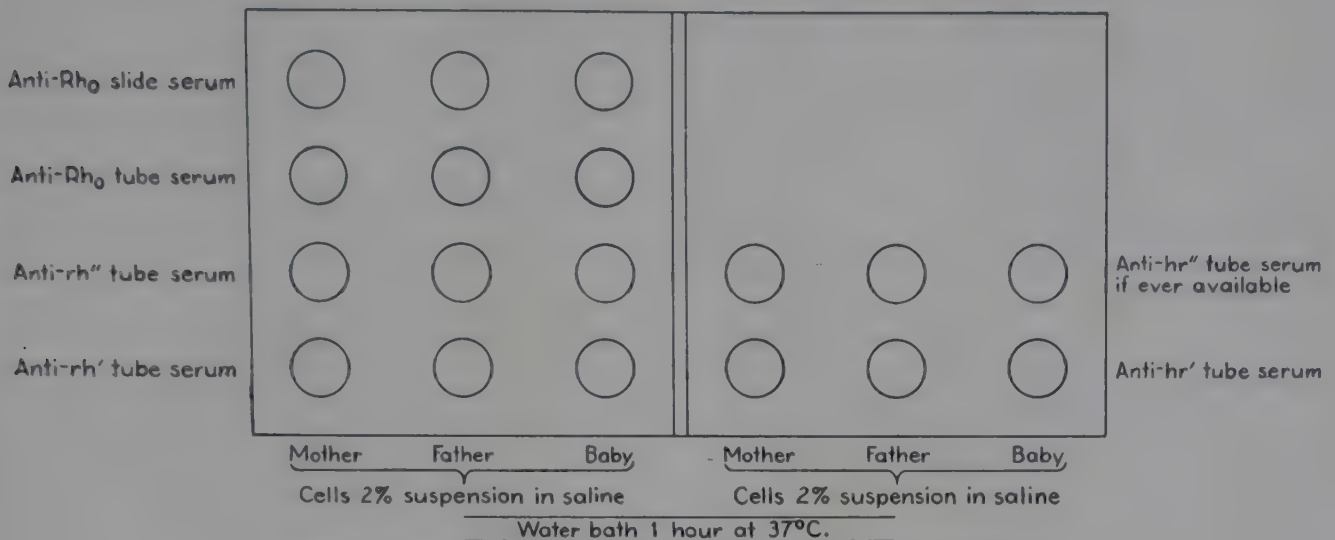
- | | |
|--------------------------------------|---|
| (a) 1 drop of saline-suspended cells | plus 1 drop anti-rh' saline agglutinating serum |
| (b) 1 drop of saline-suspended cells | plus 1 drop of anti-rh'' saline agglutinating serum |
| (c) 1 drop of saline-suspended cells | plus 1 drop of anti-Rh ₀ saline agglutinating serum |
| (d) 1 drop of saline-suspended cells | plus 1 drop of anti-Rh ₀ slide test (blocking) serum |

- (e)* 1 drop of saline-suspended cells plus 1 drop of anti-hr' saline agglutinating serum
- (f)† 1 drop of saline-suspended cells plus 1 drop of anti-hr'' saline agglutinating serum (when available)

Incubate in a water bath at 37° C. for 1 hour.

Take bottom readings. See page 1058.

SET-UP FOR BLOOD GROUPING TESTS IN CASES OF DISPUTED PATERNITY



When Anti-Rh₀ tube serum gives a negative result, wash contents of tube 4 (Anti-Rh₀ slide serum) three times with saline, remove saline, shake, replace in water bath 5 minutes, add 1 drop of Coombs serum, shake, centrifuge 2 minutes at 500 to 1000 r.p.m., and read. If positive, Rh₀ is present.

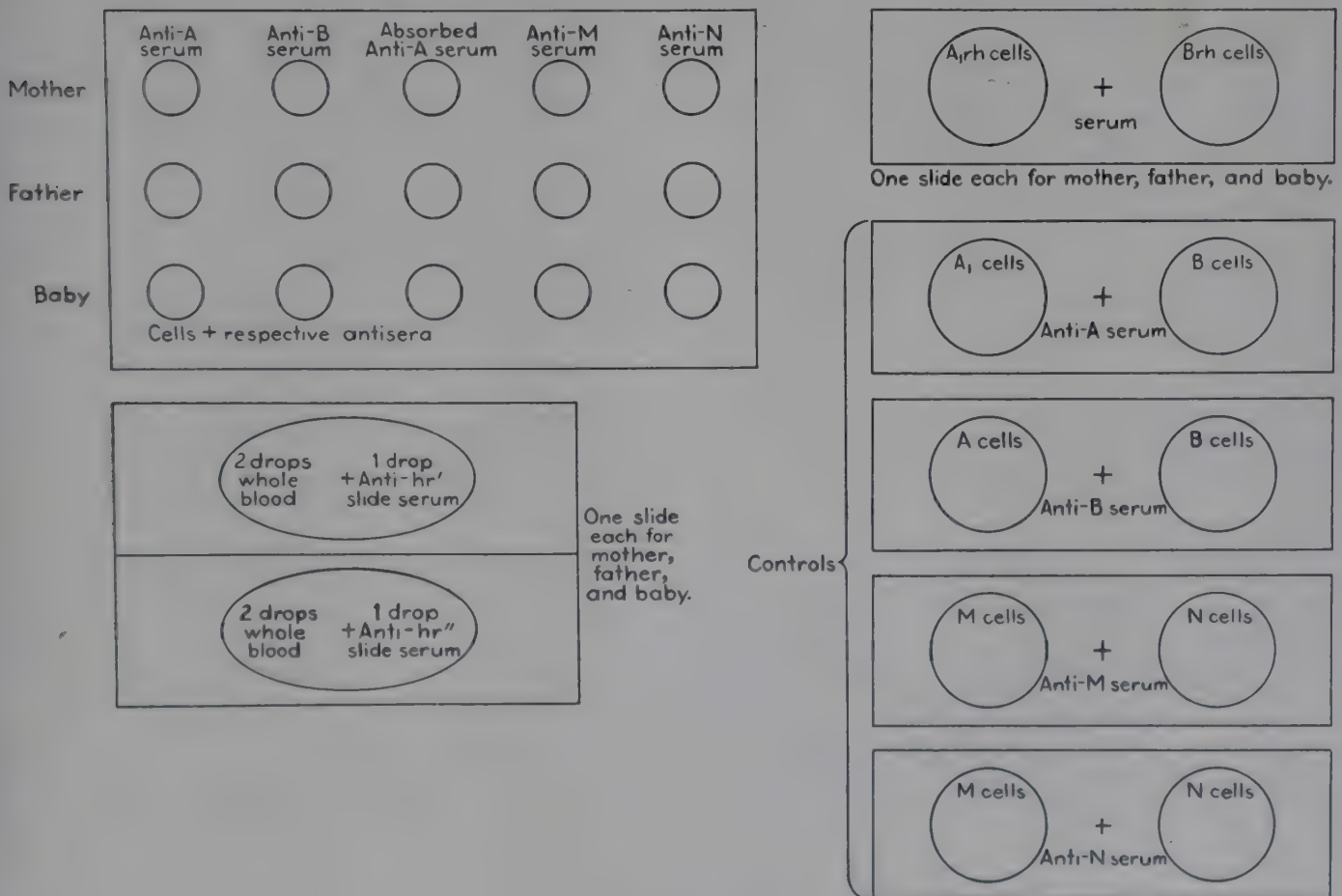


Fig. 288B.

*Not necessary when blood is rh'-negative: reaction would be positive.

†Not necessary when blood is rh''-negative: reaction would always be positive.

Read under a scanning lens of a microscope and report all clumping as positive and lack of clumping as negative.

If the results with the anti-Rh₀ saline agglutinating serum are negative, use tube 4 containing the cells plus anti-Rh₀ slide test serum. Wash three times with saline and then remove all traces of saline (see page 1058). Shake; incubate at 37° C. for 5 minutes in a water bath, and add 1 drop of Coombs serum (antiglobulin), centrifuge for 2 minutes at 500 to 1,000 r.p.m., and read the reaction under a scanning lens. Do not perform this test if the results with anti-Rh₀ saline agglutinating serum are positive because this would always be positive.

6. Make a test for the Hr factors by the slide method. Use 2 drops of whole blood (50 per cent cell suspension in their own serum or plasma) and 1 drop of the respective anti-hr' or anti-hr'' sera.*†

Mix and spread each with a wooden applicator.

Read over a lighted viewing box, preheated for 30 minutes. Rock back and forth for 3 to 5 minutes.

Add a drop of saline and rock back and forth to eliminate rouleaux formation.

Do not make the 'anti-hr' test on Rh-negative bloods, because this test would always be positive under the circumstances. Whenever the test for rh' is positive, make the test for hr'. Similarly, only when the test for rh'' is positive, make the test for hr''. All Rh-negative bloods are hr'-positive and hr''-positive.

7. Record the results according to Table 128. Draw conclusions as to phenotype and genotype according to Wiener's Table 111 on page 1053. Table 128 is given here as an example.

It must be remembered that it is never necessary to make tests for hr' if rh' is absent from the blood. Similarly, it is not necessary to make tests for

TABLE 128

CELLS PLUS RESPECTIVE SERA									
	ANTI-A SERUM	ANTI-B SERUM	ABSORBED ANTI-A SERUM	ANTI-M SERUM	ANTI-N SERUM	ANTI-rh' SALINE AGGLUTI- NATING SERUM	ANTI-rh'' SALINE AGGLUTI- NATING SERUM	ANTI-Rh ₀ SALINE AGGLUTI- NATING SERUM	ANTI-Rh ₀ SLIDE SERUM
Mother	+	-	-	+	-	-	-	-	-
Father	-	+		-	+	+	+	+	Not neces- sary
Baby	+	-	-	+	+	-	+	+	Not neces- sary

	SERUM PLUS A ₁ CELLS	SERUM PLUS B CELLS	CELLS PLUS ANTI-hr'* TUBE OR SLIDE SERUM	CELLS PLUS ANTI-hr''* TUBE OR SLIDE SERUM	BLOOD GROUP
Mother	-	+	+†	+	A ₂ M rh
Father	+	-	+	+	B N Rh ₂ Rh ₀ (see Table 111)
Baby	-	+	+	+	A ₂ MN Rh ₂ rh (see Table 111)

*When available.
†Not necessary. All Rh-negative bloods are invariably hr' and hr'' positive.

hr'' if **rh''** is absent from the blood. If, however, the blood is **rh'** positive, tests must be made for the **hr'** factor, and if the blood is **rh''** positive, tests must be made for the **hr''** factor.

The following combinations of genotypes are possible in this case:

Mother: A_2A_2 or A_2O , MM , rr

Father: BB or BO (in this case, if he is the father of this child he must be BO), NN , R^zR^o or R^1R^2 or R^zr , R^1r'' , R^2r' , R^or'' .

Baby: A_2A_2 (or A_2O if this man is the father of the child), MN , R^2R^o or R^2r or R^or'' .

against

REPORT ON
BLOOD GROUPING TEST

To the

County of

Pursuant to an order duly entered in the above-entitled action bearing date, the.....day of
.....19 , I proceeded to examine the following named persons:

NAME	IDENTIFICATION

Fig. 289.—Form for reporting results of blood grouping tests in cases of disputed paternity.
(Courtesy Alexander S. Wiener.)

Results:

Blood of	Group and Subgroup	M-N Type	Rh-Hr Type
1.			
2.			
3.			
4.			
5.			

Interpretation:

Respectfully submitted,

ALEXANDER S. WIENER, M.D.

LAWS OF HEREDITY

- I. The Blood Groups (O, A, B, and AB)
 - 1. The agglutinogens A and B cannot appear in the blood of a child unless present in the blood of one or both parents.
 - 2. Group AB parents cannot have group O children, and group O parents cannot have group AB children.
- II. The Blood Types (M, N and MN)
 - 1. The agglutinogens M and N cannot appear in the blood of a child unless present in the blood of one or both parents.
 - 2. Type M parents cannot have type N children, and type N parents cannot have type M children.
- III. The Subgroups of Group A (A₁ and A₂) and Group AB (A₁B and A₂B)
 - 1. No child can belong to subgroup A₁ or subgroup A₁B unless one or both parents belongs to one of these subgroups. For example, two parents both of subgroup A₂ cannot have a child of subgroup A₁.
 - 2. Parents of subgroup A₁B cannot have children of subgroup A₂, and parents of subgroup A₂ cannot have children of subgroup A₁B.
 - N. B. When a man is excluded by these tests, it is highly probable, though not absolutely certain, that he is not the father of the child in question.
- IV. The Rh Blood Types [rh, rh', rh'', rh'rh'' (or rh₇), Rh₀, Rh₁ (or Rh₀'), Rh₂ (or Rh₀'') and Rh₁Rh₂ (or Rh_z)]
The Hr Factors: Rh₁Rh₁ = Rh₁ hr' -, Rh₁rh = Rh₁ hr' + rh'rh' = rh' hr' -, rh'rh = rh' hr' +
Rh₂Rh₂ = Rh₂ hr'' -, Rh₂rh = Rh₂ hr'' + rh''rh'' = rh'' hr'' -, rh''rh = rh'' hr'' +
 - 1. Factors Rh₀, rh', rh'', hr' and hr'' cannot appear in the blood of a child unless present in the blood of one or both parents.
 - 2. Parents who are rh' negative cannot have children who are hr' negative, and hr' negative parents cannot have rh' negative children.

References: Wiener, A. S.: Blood Groups and Transfusion, 3rd edition, 1943. Wiener, A. S.: Amer. J. Human Genetics 2:177-197, 1950. Wiener, A. S.: Bull. World Health Org. 3:265-278, 1950.

Fig. 290.—Form for reporting blood groups and interpretation in cases of disputed paternity. (Courtesy Alexander S. Wiener.)

MEDICOLEGAL ASPECTS OF BLOOD TRANSFUSION

This chapter cannot be closed before considerable attention is paid to an important report by the Committee on Medicolegal Problems of the American Medical Association.¹ This Committee was composed of Alexander S. Wiener, Chairman, and David N. W. Grant, Lester J. Unger, and William G. Workman. They represent a group of outstanding serologists with a vast experience in this field. The following remarks are based largely upon this Report, and while at this time (1956) the material is authoritative, one cannot predict from the rapid strides that are now being made whether or not in the future they will necessarily be modified.

The report states that approximately one death in 1,000 to 3,000 transfusions has been reported. This makes blood transfusion as important a cause of death as appendicitis or anesthesia.

Errors are frequently made during emergency periods when the regular technician is not at hand, where a certain number of errors are inevitable. They advise that all hospitals should carry liability insurance and that medical schools should devote more attention to blood grouping in their curricula.

Unfavorable results have occurred because blood grouping sera have been inadvertently interchanged; also, when two patients require transfusions at the same time and the bottles of blood intended for them have been interchanged. In a few cases, errors have resulted because the wrong pilot tube was affixed to a bottle of blood. Most transfusion accidents due to mixing up of bloods can be traced to a failure to read labels. Colored grouping sera and labels have the disadvantage that the technician has a tendency to rely on his memory of the colors instead of reading the labels on the bottle of blood.

If a patient dies as a result of a transfusion of blood of an incorrect group, or suffers prolonged illness due to injury to his kidneys, the patient's family or the patient himself, if he survives, may be entitled to damages.

One of the commonest causes of hemolytic reactions is the transfusion of blood of an incompatible blood group.

Anti-A and anti-B isoantibodies in human sera are generally of low titer and avidity, and, if so, an initial transfusion of group A blood to a group O individual might be uneventful or produce only a slight reaction. As a result of such a transfusion of incompatible blood, the isoantibody titer will rise, so that if the error is repeated a fatal reaction could result.

The severity of the reaction in strongly sensitized individuals depends on the amount of blood transfused, fatalities in adults being infrequent with quantities of blood less than 300 c.c. Therefore, if this portion of each unit of blood is given slowly and the patient watched closely during that time for untoward symptoms, it may be possible to interrupt the transfusion in time, should a reaction develop. This cannot occur in patients under anesthesia.

¹J.A.M.A. 151: 1435, 1953.

Subgroups of A

The A_2 agglutinin is characterized by its weaker reaction with anti-A serum. The reactions are especially weak in the presence of agglutinin B, so that blood of subgroup A_2B has often been incorrectly classified as group B. The low avidity of most anti-A sera for A_2B blood which is responsible for errors in blood grouping also protects the patient receiving such incompatible blood from any dangerous reaction.

The Rh Factor

For blood transfusion practice, it is sufficient to limit the tests on patients to only one of the Rh-Hr factors, Rh_0 , the factor which corresponds to the one originally discovered with the aid of anti-rhesus immune animal sera. Generally, the initial transfusion of Rh-positive blood into an Rh-negative recipient is symptomless. In individuals who have been previously sensitized as a result of an injection of Rh-positive blood or a pregnancy with an Rh-positive fetus, even the first transfusion of Rh-positive blood can cause a severe reaction.

By carrying out cross-matching tests by the modern technics, namely, the saline, the conglutination, and the antiglobulin methods, incompatibilities with respect to the other factors of the Rh-Hr systems can be detected should isosensitization be present.

Persons with blood possessing an Rh_0 variant may be incorrectly classified as Rh-negative. A transfusion of blood containing an Rh_0 variant into an Rh-negative recipient can give rise to isosensitization.

Under certain conditions, Rh-negative blood may seem to react as Rh-positive. For instance, if bloods from pregnant women are tested by the slide technic, rouleaux formation, which often occurs in pregnancy, may be mistaken for clumping. In acquired hemolytic anemia, the red blood cells may be coated with autoantibodies, which may be responsible for clumping in tests carried out by the conglutination method.

In most cases of transfusion reactions where Rh-positive blood is transfused to Rh-negative individuals, the error can be traced to some laboratory error rather than a peculiarity in the blood itself. A hemolytic reaction occurring after a transfusion of Rh-positive blood into an Rh-negative patient is usually evidence of negligence.

The selection of donors is no longer a simple process. Any blood bank and hospital where blood transfusions are carried out should have a specially trained technician to handle the work under qualified medical supervision. The dangers of transfusion have been summarized by Unger² and Wiener.³

Universal Donors

While transfusion of group A blood into a group O recipient may cause a serious reaction, the transfusion of group O blood into a group A recipient is generally harmless. In the former case, the small volume of incompatible

²Unger, L. J.: New York M. J., May 1954.

³Wiener, A. S.: J. A. M. A. 150: 1301, 1954.

donor's cells combines with the isoantibodies present in a much larger volume of the recipient's plasma, while in the latter instance a limited amount of incompatible donor's plasma is diluted in the recipient's circulation and absorbed by the great mass of the patient's red blood cells. Blood containing incompatible agglutinins is much less dangerous than blood containing incompatible agglutinogens.

Transfusions of group O blood into individuals of other groups may be dangerous. Hemolysis may follow such transfusions when the donor has isoagglutinins of high titer in his serum. While injection of high-titered group O blood into individuals not belonging to group O may produce an acute hemolytic reaction, the symptoms are usually somewhat different from those which occur when incompatible red blood cells are injected. The use of group O blood as "universal donor" blood should be restricted to emergencies where there is not enough time to determine the group of the recipient reliably.

If it is not always possible to carry out all the tests necessary in cases of emergency, that is, tests relative to the Rh factor, preferably low-titer group O Rh-negative blood should be used for transfusions, especially in women during the child-bearing period. One must balance the danger of hemorrhage against the danger of isosensitization. A suitable entry should be made on the patient's chart to justify the course of action taken.

The transfusion of Rh-negative blood of the proper blood group into an Rh-positive recipient is almost always safe. Careful cross-matching tests must be done to detect incompatibilities aside from those due to the **Rh₀** factor (Rh subtypes, Hr, etc.).

Other Blood Groups

The sera of certain rare individuals contain irregular isoantibodies for blood group factors other than A and B, for instance, anti-**M**, anti-**P**, and anti-*Lewis*. In addition, immune antibodies to blood factors besides Rh-Hr may be formed as a result of isosensitization, notably anti-**M**, anti-*Kell*, and anti-*Duffy*. This may cause serious hemolytic reactions.

The Committee does not believe that reactions caused by sensitizations to one of the other blood factors can properly be a cause of action for a lawsuit, provided that the physician and hospital have exercised ordinary care to protect the welfare of the patient.

Damaged Blood

Blood damaged during storage may break down rapidly in the patient's circulation and give rise to hemoglobin nephrosis. No blood should be used if, after inspection, hemolysis is suspected.

Red blood cells are sensitive to freezing and overheating. Dry ice must not be used. No attempt should be made to warm blood prior to or during its administration. Blood stored too long or improperly can give rise to transfusion hemolysis. The National Institutes of Health permit an expiration date of 21 days for blood stored in citric acid-dextrose solution.

Blood should be kept cold from the moment it is collected until it is used. It should be stored in a refrigerator set preferably at 4° to 6° C. The temperature of the refrigerator must be recorded at least once a day.

Transmission of Disease

One of the most serious dangers is transmitting homologous serum hepatitis to the patient. This may occur once among 200 patients receiving transfusions, and may be responsible for the patient's death once among 6,000 transfusions. The danger is graver following plasma transfusion.

Homologous serum hepatitis was observed during World War II as a result of the extensive use of pooled dried plasma when treating battle casualties. Pooling increases the hazard of homologous serum hepatitis, because the presence of the virus in a single sample of blood plasma will serve to contaminate the entire pool. The most important difference between homologous serum hepatitis and infectious hepatitis, or catarrhal jaundice, is the mode of transmission. There is no simple test by which the carrier of the virus can be recognized, and the presence of the virus in the plasma pool can be detected only by testing it on human volunteers.

The National Institutes of Health have required that pooled plasma be irradiated before distribution, and this ruling is still in effect. Recent observations, however, indicate that such irradiation may not always be effective. Irradiation is not applicable to whole blood. The problem of homologous serum hepatitis has not yet been solved.

Syphilis

Transmission of syphilis by transfusion is rarely heard of any longer.* This is mainly due to the development of more sensitive tests for the detection of the disease in prospective donors, while another effective factor in preventing the disease is the widespread use of bank blood. According to this report: "Since the agent of this disease cannot live more than a few days outside the body, even at refrigerator temperature, the danger of transmitting syphilis is virtually nonexistent when blood or plasma aged for at least three days is used for transfusions." *No individual with positive serologic tests for syphilis or positive history of the disease should be used as a donor.*

Malaria

Transmission of malaria by transfusion is a difficult problem in regions where the disease is endemic, but until recently it was not an important problem in the United States. For obtaining fresh blood for transfusion, individuals who have had malaria or who have received suppressive therapy for malaria while residing in a malarious region should not be used as donors for a period of at least two years following the last proved attack of discontinu-

*Elsewhere in this chapter (pages 1086 ff.) will be found the author's own views on this subject. His views are based upon experiences, not with blood bank blood, but with fresh blood from individual donors.

ance of suppressive therapy. The *Plasmodium malariae* can survive in a refrigerator for as long as five days, so that bank blood less than five days old might be infectious.

Other Infectious Diseases

Other infectious diseases, such as measles and influenza, have been transmitted by blood transfusion, when the donor was in the prodromal stage of the disease at the time his blood was used for transfusion. All prospective donors should be examined to detect the presence of infectious disease.

Contaminated Blood

Bacteriologic surveys have shown that as many as 5 to 10 per cent of bank blood specimens are contaminated with bacteria. No harm results usually, because the majority of microorganisms present are gram-positive bacteria relatively low in pyrogenicity, do not grow at refrigerator temperature, and are generally nonpathogenic. Some bacteria grow at refrigerator temperature.* Transfusion of such blood has resulted in death of the patient. At this time it is not considered advisable to add any antiseptic to blood in an attempt to prevent bacterial growth. Prime reliance must be placed on stringent aseptic technic when collecting and storing blood.

Other Accidents Following Transfusion

Embolism.—During storage of blood or plasma, precipitation of fibrin gradually occurs, and if such blood or plasma is transfused unfiltered, multiple embolization into the lung bed may occur, or, if the recipient has a cardiac septal defect, into the viscera or brain. All bank blood and stored blood should be filtered during transfusion.

Rapid transfusion of blood by pumping air under pressure into the blood reservoir has caused a number of deaths from air embolism. This type of injection should be condemned.

Allergic Reactions.—About 1 per cent of blood transfusions are followed by reactions of an allergic type. These reactions are mild and consist merely of urticaria. Angioneurotic edema is a more serious allergic manifestation which may be dangerous if it involves the larynx. It is best not to use allergic individuals for blood donors.

Pyrogenic Reactions.—Pyrogenic reactions have been caused by growth of bacteria in the water used for preparing solutions, or in moisture adherent to the apparatus, before they are sterilized.

Methods have been developed for preparing pyrogen-free solutions and apparatus, which are readily available commercially. (See pages 1123 ff., Pyrogenicity.) Therefore, pyrogenic reactions should no longer be an important problem.

Overloading.—Too rapid transfusion or transfusion of too much blood may cause death from congestive cardiac failure or cerebral hemorrhage. A

*Stevens and associates (Ann. Int. Med. 39: 1228, 1953) reported a fatal transfusion reaction due to cold growing *Pseudomonas* sp. in stored blood.

certain number of deaths from too rapid transfusions still occur, especially when transfusions are given to infants.

Citrate Reactions.—The sodium citrate which is introduced during transfusion is rapidly metabolized and excreted. The amount of citrate administered with 500 c.c. of blood is harmless to the adult. When massive transfusions are given in a short period of time, sodium citrate may accumulate in the blood stream, so that signs of hypocalcemia may result, namely, a drop in blood pressure and tetany. This may happen when adults with massive hemorrhage require more than a liter of blood. If 10 c.c. of a 10 per cent solution of calcium gluconate are injected slowly intravenously after every 1,000 c.c. of citrated blood, hypocalcemia may be prevented. When performing exchange transfusions on erythroblastotic babies, some transfusionists inject 1 c.c. of 10 per cent calcium gluconate after every 100 c.c. of citrated blood; others meet the problem by removing part of the citrated plasma from the blood to be used for the transfusion.

Intramedullary Transfusion

In adults, bone marrow transfusion is made through the sternum. In infants and small children where the sternum is too thin, the tibial route is used. The Committee believes that the intramedullary route for ordinary transfusions is not recommended, because of the danger of fat embolism, especially in children. The procedure has resulted in osteomyelitis, and fractures of the tibia. Intramedullary transfusion has its main application in instances of extensive burns or in patients with skin diseases, which makes intravenous therapy difficult.

Other Routes

Transfusion into the longitudinal sinus in inexperienced hands may be dangerous because the blood may be injected extradurally. Nobody any longer advocates the use of intraperitoneal or penile routes.

How to Protect the Blood Donor

If a donor gives blood too frequently, there is likelihood of secondary anemia, a danger greater in women than in men. Donors should not be permitted to donate a pint of blood more often than every 6 weeks or until the blood attains a hemoglobin concentration of at least 12.5 gm. per 100 c.c., or a specific gravity of 1.053 or higher.

A sterile needle, preferably disposable, should be used when obtaining blood for hemoglobin concentration to avoid transmission of hepatitis virus.

Fainting.—Donors often faint when blood is taken or when they see other blood taken. Aside from the psychic aspect, the cause is sudden reduction in blood volume. The incidence of fainting can be reduced by screening out unsuitable donors by physical examination to exclude those with low blood pressure or a low hemoglobin level, or with a small body size. A donor who has been without food for a long time or who makes his donation after coming off a night shift may be a candidate for fainting. They should be given

a light snack before taking their blood. After the donation, the donor should be kept in a supine position until he has apparently recovered. The nervous donor should have his attention diverted from the procedure by pleasant conversation. The room should be light and dry and smoking should not be permitted in it or in the resuscitation room. On hot days, the donors may be given some isotonic sodium chloride solution before the bleeding. If the donor faints, keep the head low, loosen the clothing, and provide with fresh air. In rare cases intravenous infusions of sodium chloride may be required.

Syncope occurs sometimes after the donor has left the hospital. He may fall and lacerate his scalp or even fracture his skull, or become involved in a serious accident if he falls in the street. Donors involved in hazardous occupations should be advised not to return to work for at least 12 hours.

Thrombosis and Infections.—In rare cases, thrombophlebitis or abscess formation has occurred at the site of the puncture. It is wise for the blood bank physicians to protect themselves with suitable insurance to prevent a lawsuit.

Donation of a pint of blood by a healthy adult is safe.

Preparation of Blood Diagnostic Sera

Blood grouping tests are possible only when potent and specific diagnostic sera are available.

Men are used more often than women for the preparation of diagnostic sera. No Rh-negative woman should be immunized with Rh-positive blood to prepare anti-Rh sera if there is any chance of her becoming pregnant later on. Similarly immunization of a group A woman against agglutinin B or vice versa might cause obstetric complications. More common sera, such as anti-**A**, anti-**B**, and anti-**Rh₀**, can be prepared by immunization of male volunteers, but this technic is not applicable to weaker factors such as **rh'**, **rh''**, **hr''**, *Kell*, and *Duffy*. To prepare anti-**A** and anti-**B** sera, a number of different immunization procedures are used.* Intravenous injection of blood group specific substances prepared from horse and pig stomach carries the possibility of anaphylactoid reactions in allergic subjects. This can be avoided by using deproteinized preparations and by administering them intramuscularly instead of intravenously. Some workers also use human blood to prepare anti-**A** and anti-**B** sera. Some subjects complain of pain along the course of the vein during the injection, or have a mild rise in temperature later on. The former can be avoided by diluting the blood with sodium chloride and injecting it more slowly, the latter by having the donor take 5 grains or 0.32 Gm. of acetylsalicylic acid at the time of the injection or by reducing the dosage. Look out for the danger of homologous serum hepatitis.

Before injecting blood into any volunteer, explain the purpose. The sensitized Rh donor should be given a suitable identification card. He should be warned that only Rh-negative blood should be used for any transfusion he may require in the future. A signed release should be secured from the donor.

*Wiener, A. S., Samwick, A. A., Morrison, M., and Cohen, L.: *Exper. Med. & Surg.* **11**: 267, 1953.

Occasionally, volunteers immunized against the Rh factor have developed antibodies against other antigens, most frequently agglutinin M. This can be avoided by testing all subjects for the M-N types, and using only type N, Rh-positive blood for Rh-negative donors of type N.

Physicians are urged to enlist the cooperation of patients whose plasma contains rare blood group antibodies, so that their blood can be used for the preparation of diagnostic sera. Insurance companies specialized in malpractice insurance can aid the work by providing riders on policies of physicians specializing in this field.

Every hospital should have one or more qualified individuals responsible for the transfusions performed there. These should see that the residents are properly trained to perform transfusions, and should be available to advise and assist them when problems of unusual difficulty arise. When nurses or technicians are used in the blood banks for blood withdrawal, this should be under the direct supervision of a qualified physician.

CHAPTER VI

GASTRIC ANALYSIS

GASTRIC CONTENTS

The clinical laboratory is concerned frequently with the determination of the chemical analysis of so-called "gastric contents." "Gastric contents" in this sense means material which remains in the stomach one hour after a classical test meal has been administered. The test meal which has been in use for many years is the so-called "Ewald Breakfast," later to be described. The purpose of placing a sample meal in the stomach is to determine after one hour just how gastric physiology has taken place during this period. Normal gastric digestion is at its height one hour after the administration of the meal.

Reviewing the physiology of gastric digestion, it is known that the secretion of gastric juice begins soon after food is placed in the stomach. It is a transparent, light-colored, yellow fluid which is acid in reaction and contains about 1 per cent of solids. The specific gravity of gastric juice is very low, varying from 1.001 to 1.010. The principal phenomenon that goes on in gastric digestion is the splitting of the protein constituents of food by pepsin in the presence of hydrochloric acid. The action of rennin on milk is also of importance. It is to be noticed that previously emulsified fats are acted upon by lipase, which is a fat-splitting ferment. Pepsinogen and renninogen are also secreted by the gastric glands and are converted into pepsin and rennin by the action of hydrochloric acid. Hydrochloric acid combines loosely with the proteins and is called "combined" hydrochloric acid. That part of hydrochloric acid which is formed after the proteins have been converted into acidmetaprotein is designated "free" hydrochloric acid.

At the time the gastric contents are removed, namely, one hour after the meal has been taken, normally the contents consist of water, hydrochloric acid, combined hydrochloric acid, pepsin, rennin, mineral salts, acid phosphates, and the remnants of undigested food. It also contains various products of digestion in solution. Under pathologic conditions, these materials are present in greater or less extent and sometimes some of them are absent. Under certain conditions, lactic acid is present. Microscopically are seen structures such as blood, bacteria, and abnormal cells.

FACTS ABOUT GASTRIC SECRETION

The stomach normally secretes hydrochloric acid, pepsin, rennin, and mucin. It is important for the laboratory to assist in estimating the degree and activity of the secretory function. Gastric acidity is an index to the concentration of the protein-splitting enzyme pepsin, and the milk-clotting enzyme rennin. There are two kinds of mucin present in gastric secretion, a soluble mucin which plays an important part in buffering the acid, and, in addition, the glairy mucus that is not concerned with this function.

According to Meakins* the amount of increased gastric secretion is best determined in a fasting state and at this time the normal stomach should contain less than 100 c.c. of more or less opaque liquid which, although acid to phenolphthalein, should not contain more than a low (under 10 per cent) concentration of free hydrochloric acid. Greater quantities than 100 c.c., and particularly if the amount of free hydrochloric acid is high, are significant of hypersecretion. Decreased secretion of acid is of considerable diagnostic importance in organic gastric lesions and systemic diseases and such a decrease even to absence of hydrochloric acid is a common accompaniment of carcinoma of the stomach. However, since it occurs in from 10 to 15 per cent of normal people it cannot be taken too seriously unless substantiated by other evidence of local disturbance. Meakins called attention, further, to lowered hydrochloric acid, or its absence, as a prominent sign in the diagnosis of certain diseases of the blood, particularly pernicious anemia. The presence of free hydrochloric acid practically eliminates the diagnosis of pernicious anemia.

Meakins stated that gastric digestion depends almost entirely upon the presence of pepsin which is active in acid medium, but the determination of peptic activity is laborious and is seldom indicated. He believes that in rare cases where there is an entire absence of acid secretion, determinations of peptic activity may be advisable.

METHOD OF OBTAINING STOMACH CONTENTS

Two kinds of tubes may be used in this technic. One is the Rehfluss tube, the other the regulation semistiff, red rubber tube. Some prefer the former, others, the latter. The Rehfluss tube consists of a small, metal oval tip attached to a very small caliber rubber tube. The tip is designed both for obtaining stomach contents and duodenal contents; i.e., it will slip through the pylorus into the duodenum after a certain period of time with the patient in correct position, lying on the right side. Most operators prefer the first kind of tube.

The tubes are, of course, cleaned thoroughly and then placed in a basin of ice water before use. The patient should be gowned and in a sitting position, and the neck should be free.

The cooperation of the patient is very important. He should be told by the operator just what is expected of him and what is about to transpire. He should be assured of the lack of pain and discomfort, provided he cooperates by keeping his mouth wide open. He should also be told to breathe as naturally as possible, and to "swallow" the tube as soon as he is requested to do so. With the mouth wide open and the head back a trifle, the head fixed in a steady position by the left hand of the operator, the right hand of the operator, without placing any fingers in the mouth, inserts the point of the tube directly in the middle line over the base of the tongue. The operator rapidly passes the tube into the posterior part of the mouth, almost "throwing" it in. The patient is now directed to swallow. At the same time, the tube is pushed along gently and firmly with each swallow. Care must be taken, of course, that the tube, when placed over the base of the tongue, does

*Meakins, J. C.: *The Practice of Medicine*, St. Louis, 1950, The C. V. Mosby Co.

not go into the trachea. This is easily avoided by practice. Above all things, the tube should not be oiled or lubricated since this material often causes the patient to vomit. There are some persons with unduly sensitive posterior pharyngeal mucosae in whom the slightest irritation will cause vomiting. It might be well in these cases to paint the mucosa lightly with cocaine; however, this is seldom necessary. During the entire time that the patient is swallowing, he must breathe regularly with his mouth wide open. Provided there is no obstruction, the tube will glide rapidly into the stomach.

As soon as the tube is in the stomach, as judged by the mark on the tube, the outside end is lowered so that it will be below the level of the end of the stomach; a siphon is thus created; the material will flow out. If there is any difficulty, a clean glass 20 c.c. syringe is attached to the outside end and suction is used. It is sometimes necessary to pull the tube gently in and out so as to remove any blocked food particles which may interfere with emptying the contents. If, in any way, failure occurs in inserting the tube, the patient must be assured that this sometimes happens and is not unusual, and that it is often necessary to try again. The psychological influence of the patient in this procedure is very important. There should be no undue movement on the part of the operator or any signs of concern manifested by any person, so the patient's psyche will in no way be disturbed. The distance from the teeth to the cardiac orifice of the stomach is about 40 cm.

If the unskilled operator has any idea at the beginning of the operation that the tube has gone into the larynx instead of into the esophagus, this idea may be dispelled or confirmed by holding the end of the tube to the ear and listening. If the intake and exhalation of air can be heard, the tube must be withdrawn. The tube does not go into the trachea ordinarily, because the opening quickly closes at the approach of a foreign body of this kind, thus throwing the point of the instrument out in the pharynx. If there is considerable disturbance and severe dysphagia and continued strong convulsive coughing during the beginning of the placing of the tube, this indicates that the tube is in the fossa pyriformes at the side of the larynx and the tube is rolled up in a spiral manner at this point. It must be withdrawn and started again. If it is not taken out immediately, it will suddenly be regurgitated after it is drawn up into the mouth. The principal point in the whole procedure is to start the tube directly in the median line, under the eye of the operator.

As noted before, in cases of abnormal sensitiveness the painting of the pharynx with 1 per cent cocaine is necessary.

After the stomach contents have been removed, the tube should be pinched securely with the fingers and rapidly withdrawn. This pinching of the tube holds any foreign body that may be in the tube securely during the process of withdrawal and thus prevents the dropping of foreign bodies into the posterior pharynx and possible entrance into the trachea.

The stomach contents are received in a clean, sterile 500 c.c. glass beaker.

Another method, instead of passing the tube orally, is to pass a small Rehfuß tube through the nostril, and follow the same precautions as above.

Tubeless Gastric Analysis

Segal, Miller, and Morton¹ advocated a method of determination of the hydrochloric acid content of gastric juice which does not require the passing of a stomach tube. This technic depends upon the power of the hydrogen cation of the free hydrochloric acid of the gastric juice at a pH of 3.0 or less to displace the quininium cation from the quininium-resin indicator compound; the quinine and hydrochloride now formed are rapidly absorbed from the small intestine. Quinine is excreted in the urine, where it appears within 15 minutes of ingestion of the resin. The quinine is readily estimated quantitatively in the urine by a simple method based on its fluorescence in ultraviolet light. Segal's quininium-resin indicator is available as a commercial product under the trade name of "Diagnex."²

THE DIAGNEX TEST FOR ACHLORHYDRIA

This test has been extremely reliable in the detection of gastric achlorhydria. The test is qualitatively reliable for the detection of free hydrochloric acid, but the concentration of the free acid present cannot be accurately predicted on the basis of the present grading system.

Powers, Carballo, and Bolt³ concluded that urinary quininium determinations in 76 patients correlated well with the disease state present, and compared favorably with the tube method of gastric analysis in the majority of cases. The method should prove of value as a screening test in the detection of patients with achlorhydria.

The test is indicated in those patients who are unable to tolerate the standard gastric tube, either because of psychogenic or organic illness which precludes the use of the gastric tube. It is extremely valuable in patients with partial gastrectomy or gastroenterostomies because of the unreliability of the gastric tube method in such cases. However, it must be remembered that, as with the uropepsin determination, the use of this test in differential diagnosis of gastrointestinal lesions is limited. A negative quininium test is strong evidence against the diagnosis of benign peptic ulcer. A 1+ to 4+ reaction is of little diagnostic value in the individual case.

Preparation of the Patient.—

The patient must omit taking vitamin preparations on the day preceding the test day. Issue the following instructions to the patient:

1. Do not eat after midnight.
2. Urinate on arising, and discard the urine.
3. Do *not* eat breakfast. Empty the contents of the capsule in the small packet (of Diagnex) into $\frac{1}{2}$ glass of water, stir, and drink. Drink another $\frac{1}{2}$ glass of water or cup of coffee or tea, *without cream, milk, or sugar*.
4. One hour later, or as soon after the one-hour interval as possible, urinate and save this urine (entire quantity) in bottle labeled "Urine Control."
5. Empty the granules in the large packet into $\frac{1}{4}$ glass of water, stir well and drink. Do not chew the granules which do not dissolve. Fill the glass again one-fourth full with water and drink.

¹Proc. Soc. Exper. Biol. & Med. 74: 218, 1950.

²Prepared by E. R. Squibb & Sons, New York.

³J. Lab. & Clin. Med. 43: 3, 1954.

6. At the end of two hours, urinate and save the entire specimen. Empty the bladder completely. If urination ahead of the scheduled time is necessary, add to that passed at the end of the two hours. Mark this 2-hour specimen "Test Urine."

After step 6, the patient may proceed with breakfast.

Technic.—

No urine must be collected after two hours. If the two-hour specimen does not exceed 300 c.c., the determination is carried out as indicated in the instructions. When the urine volume exceeds 300 c.c., the total volume is measured and a 30 c.c. aliquot of this is tested for quinine without dilution. Corrections for the use of this aliquot are $\frac{\text{total urine volume}}{300} \times \text{quantity of quinine in the aliquot}$. This will give the total quinine in the voided urine.

If the physician prefers, histamine may be used as the gastric stimulant and the Diagnex granules may be administered about 45 minutes after administration of histamine.

Carry out the technic on both the control urine and the test urine.

Dilute the entire quantity of each urine to 300 c.c. with distilled water.

To a 30 c.c. portion of this dilution in a separatory funnel, add 0.5 c.c. of N/2 (0.5N) sodium hydroxide.

Extract with 15 c.c. of ether, shaking only gently to prevent emulsification. If necessary, add a few drops of 95% ethyl alcohol to obtain sharp separation of the two layers.

Remove 8.2 c.c. of the ether layer.

Extract with 5 c.c. of N/10 sulphuric acid, inverting 12 complete times. Let it separate and take 5 c.c. of the bottom layer for the test.

Examine this aqueous extract for fluorescence, using a suitable source of ultraviolet light.

A special small separatory funnel, marked off in appropriate places, may be obtained for this test.* If it is used, proceed as follows:

Pour the urine, previously diluted with distilled water to 300 c.c., into the funnel up to mark I.

Add 0.5 c.c. of N/2 sodium hydroxide.

Add ether to mark II.

Invert the funnel gently 5 or 6 times.

Allow to stand for separation of the layers, adding a few drops of 95% ethyl alcohol if necessary to achieve sharpness.

Drain off and discard the contents of the funnel until the top of the ether layer is at mark III.

Add N/10 sulphuric acid to mark IV.

Shake about 10 times.

Allow the layers to separate.

Drain off 5 c.c. of the lower layer for the fluorescence examination.

Measurement of Fluorescence.—

If a photoelectric fluorophotometer is available, the urinary quinine level may be estimated directly from a previously determined standard curve.

If other means of ultraviolet irradiation are used, such as the Menlo Fluoretor,† the Blak-Ray Lamp, Model X-4,‡ or the lamp made by U.V. Comparator,§ estimation must be made by visual comparison with standard solutions containing known amounts of quinine. Since such quinine solutions are of only limited stability, they should be made fresh on the day of use. If a lamp of the Blak-Ray type is used, the observation should be made in a dark room.

*Will Corporation, Rochester 3, N. Y., supplies the separatory funnel.

†Made by Menlo Research Laboratory, Menlo Park, Calif.

‡Made by Ultra-Violet Products, Inc., South Pasadena, Calif.

§710 N. Stricker St., Baltimore, Md.

Standard Solutions.—

Standard solutions may be prepared by using Squibb Diagnex standard tablets.

Each tablet contains quinine hydrochloride equivalent to 1.5 μg of quinine base. The series of standard solutions is made by dissolving 5 of these tablets in a volume of 25 c.c. of N/10 sulphuric acid and diluting with N/10 sulphuric acid in 6 separate test tubes according to Table 129.

TABLE 129

VOLUME OF SOLUTION	AMOUNT OF N/10 SULPHURIC ACID TO BE ADDED (TO MAKE 5 C.C.)	AMOUNT OF QUININE IN STANDARD	EQUIVALENT TO	AMOUNT OF QUININE IN URINE
1. 5.0 c.c.	0.0 c.c.	1.5 μg	=	30 μg
2. 4.15 c.c.	0.85 c.c.	1.25 μg	=	25 μg
3. 3.33 c.c.	1.67 c.c.	1.0 μg	=	20 μg
4. 2.5 c.c.	2.5 c.c.	0.75 μg	=	15 μg
5. 1.67 c.c.	3.33 c.c.	0.5 μg	=	10 μg
6. 0.83 c.c.	4.17 c.c.	0.25 μg	=	5 μg

Or the standards may be made as follows:

Prepare a **stock solution of quinine sulphate** by dissolving 12 mg. of quinine sulphate in 100 c.c. of N/10 sulphuric acid in a volumetric flask (1 c.c. = 0.1 mg. quinine). The stock solution keeps indefinitely, but fresh standards for comparison must be prepared every few days.

Fresh standards are prepared by diluting 0.3 c.c. of the stock solution with N/10 sulphuric acid to a volume of 15 c.c. This solution contains 2 μg of quinine per c.c.

Place in 6 separate test tubes and dilute with N/10 sulphuric acid as in Table 130.

TABLE 130

VOLUME OF SOLUTION (2 μg QUININE PER C.C.)	AMOUNT OF N/10 SULPHURIC ACID TO BE ADDED (TO MAKE 5 C.C.)	AMOUNT OF QUININE IN STANDARD	EQUIVALENT TO	AMOUNT OF QUININE IN URINE
1. 0.75 c.c.	4.25 c.c.	1.5 μg	=	30 μg
2. 0.62 c.c.	4.38 c.c.	1.25 μg	=	25 μg
3. 0.5 c.c.	4.5 c.c.	1.0 μg	=	20 μg
4. 0.37 c.c.	4.63 c.c.	0.75 μg	=	15 μg
5. 0.25 c.c.	4.75 c.c.	0.5 μg	=	10 μg
6. 0.12 c.c.	4.88 c.c.	0.25 μg	=	5 μg

Interpretation.—

The fluorescence of the control sample of urine should first be determined. If the result shows a fluorescence corresponding to 15 μg or more of quinine, the entire test must be disregarded. Report that the patient is excreting excessive amounts of blank fluorescent materials and that the test will have to be repeated after a week, during which intake of the interfering materials, such as quinine or related drugs, the B complex vitamins, or the steroid compounds, is to be eliminated.

If the result on the urine control corresponds to 5 to 15 μg of quinine, the findings on the test urine sample may be interpreted by the method outlined below. However, the results should be corrected for the fluorescence found in the control. This may be done by subtracting the amount of fluorescence seen in the control from the test sample. If the amount of fluorescence of the

urine control corresponds to less than 5 μg of quinine, it may be ignored, and the findings on the test urine sample may be interpreted on the basis of Table 131.

When the total for the urine is within the range of 15 to 25 μg the test should be repeated, but not for 5 to 7 days. A second result in the 15 to 25 μg range means that, while the case is not definitely one of hypoacidity, the possibility of hypoacidity is not ruled out. If the results of this second test are uncertain, the physicians may wish to do other studies such as gastric analysis and intubation to determine achlorhydria. Moreover, it may be advisable for him to use intubation to confirm his findings in every case until facility has been gained in the use of the Diagnex method.

In order to determine the presence or absence of free gastric hydrochloric acid, results on the test urine samples may be interpreted according to Table 131.

TABLE 131

MICROGRAMS OF QUININE OBSERVED IN URINE SAMPLE	FREE GASTRIC HYDROCHLORIC ACID
30 or more	Present
25 or more	Present
About 15	Absent
15 or less	Absent

NOTE: Use only distilled water and solutions which are stored in glass-stoppered bottles; prolonged contact with rubber is particularly to be avoided.

The ether should be of anesthetic grade, preferably from recently opened containers.

The reagents should be checked periodically to establish that they have not become contaminated or undergone any change which might contribute to fluorescence.

If a single urine specimen exceeds 300 c.c., transfer 300 c.c. to a flask and prepare 30 c.c. of this amount as described above, without diluting. If the amount of quinine in this sample is above 25 μg , further examination is unnecessary. If it is less than 25 μg , transfer the remaining urine above 300 c.c. to another flask, dilute to 300 c.c., and prepare for examination in the usual manner. Add the results of both examinations to obtain the total amount of quinine in the urine sample.

TEST MEAL EXAMINATION

The usual method of examination of the stomach contents is one examination obtained one hour after a test meal.

Test Meals

Test meals are given to stimulate secretion of the stomach with food. It can be stimulated with various foods, but for the sake of obtaining uniform results, certain standard test meals have been adopted.

Test meals are given in the morning because the stomach is usually empty at this time. If there is reason to believe the stomach will not be empty in the morning, it is well to wash it with water the evening before.

1. **Ewald's test breakfast** consists of 40 grams of bread (one roll or two slices of bread) and 400 c.c. of water, or weak tea without cream or sugar. It should be well masticated.

2. **Dock's test breakfast** is the same as the Ewald breakfast except that a shredded whole wheat biscuit is substituted for the bread. This substitution is carried out because the Ewald test meal introduces, with the bread, a variable amount of lactic acid and numerous yeast cells, which are completely eliminated by the shredded wheat meal. The shredded wheat biscuit contains no lactic acid.

3. **Boas' test breakfast** consists of a tablespoonful of rolled oats in a quart of water, boiled to one pint, with a pinch of salt added. This should be withdrawn in forty-five minutes to one hour after introduction. It does not contain lactic acid. When this meal is used the stomach should always be washed with water the evening before.

4. **Riegel's test meal** consists of 400 c.c. of bouillon, a broiled beefsteak (about 150-200 gm.) and 150 gm. of mashed potato. It must be thoroughly masticated, otherwise the tube may be occluded when attempt is made to withdraw the meal.

5. **Fischer's test meal** is similar to the Riegel, consisting of an Ewald breakfast plus one-fourth pound of lean, finely chopped Hamburg steak, broiled and lightly seasoned. The Fischer meal and the Riegel meal may be removed in three to four hours. They give higher acidity values than the Ewald breakfast.

6. **Histamine test** is a test which has for its object the demonstration of the ability of the stomach to secrete free hydrochloric acid under the maximum amount of stimulation. It should be done as an initial test if pernicious anemia is suspected. It should also be done as a control on any patient showing achlorhydria or hypochlorhydria with the Ewald test meal. In other words, if the gastric analysis shows an achlorhydria or hypochlorhydria after using this powerful stimulating agent, the results are much more significant than the lowered value for hydrochloric acid or the absence of hydrochloric acid after an Ewald meal. This is administered only on order of a physician.

The test should be performed with the patient under as nearly a basic condition as possible. Remove the stomach contents of a fasting patient by means of a Rehfuess tube. Then administer histamine hydrochloride or histamine phosphate hypodermically. The usual dose is 0.1 mg. per kg. of body weight. Gompertz and Cohen¹ recommend a total dose of 0.25 mg. This is the standard dose of histamine but the actual dose of histamine phosphate equivalent to 0.1 mg. of histamine is 0.19 mg. for each 10 kg. This can be most advantageously administered by injecting 0.25 c.c. of a sterile 1:1000 solution of histamine subcutaneously. Completely aspirate the stomach contents at 30-minute intervals from the time of injection, and determine the volume and total acidity of the samples. The free hydrochloric acid is the same as the total acidity.

7. **Alcohol Test of Gastric Function.**—The use of alcohol with phenolphthalein added as an indicator has been suggested as a substitute for test meals. The technic is to have the patient swallow 49 c.c. of a 7% solution of

¹Gompertz, L. M., and Cohen, W.; *Am. J. M. Sc.* 177: 59, 1929.

alcohol to which has been added 1 c.c. of 0.1% phenolphthalein and 90% alcohol. Determine the percentage of the dye in the stomach contents withdrawn from fasting stomach, by colorimetric methods. It is believed by some that the amount of dilution of the hydrochloric acid may be calculated and that the actual volume acidity of gastric juice may be obtained by this method. It is doubtful whether or not this test may be relied upon because other substances, such as duodenal contents, saliva, etc., may dilute the contents and combine with hydrochloric acid to change the dilution figure.

✓ Examination of the Stomach Contents

Preliminary Survey.—

Volume.—Normal stomach yields about 20 to 50 c.c. This may be more or less, pathologically.

Color.—Normal is colorless. It may be green from bile. Blood is characteristic when present. Color may be altered by blood or drugs.

Consistency.—Normally it is clear. It may contain mucus or undigested food particles.

Odor.—Normally odorless. In disease the odor is sour or rancid. Watch for the odor of drugs.

Sediment.—Normally none. Pathologically, pieces of mucus, food remnants, blood clots, tumor particles.

Starch.—Add a drop of Gram's iodine to the gastric contents. If starch is present, it will turn blue, if undigested. If partially digested, it will turn red.

The *reaction* of gastric contents is acid in health and in nearly all pathologic conditions. It may be neutral in some cases of gastric cancer and marked chronic gastritis, or when contaminated by a considerable amount of saliva.

A small amount of mucus is normally present, but large amounts point to chronic gastritis.

Bile is seen in very minute particles as the result of excessive straining while the tube is in the stomach. Large amounts are rarely found but if they are found, it indicates obstruction in the duodenum.

The Chemical Examination of Gastric Contents

Routine examination means a qualitative test for free hydrochloric acid and organic acid, and quantitative estimations of total acidity, free hydrochloric acid, and sometimes combined hydrochloric acid. In routine work qualitative tests are performed before quantitative tests are begun.

Topfer's Test for Free Hydrochloric Acid

(a) Qualitative.—

Reagents.—

Topfer's Reagent.—

Dimethylaminoazobenzol	-----	0.5 gm.
95% Alcohol	-----	100.0 c.c.

Technic.—

Place a few drops of gastric juice in a test tube. Add one drop of Topfer's reagent. A bright red color will appear if free hydrochloric acid is present. Organic acids produce a red color, but it is less brilliant.

(b) Quantitative.—**Reagents.—**

Topfer's Reagent (above).

N/10 Sodium Hydroxide (page 25). This may be prepared by diluting N/1 solution of sodium hydroxide 1:10.

To about 50 c.c. distilled water in a 100 c.c. volumetric flask add 10 c.c. N/1 sodium hydroxide. Dilute to 100 c.c. with distilled water.

Technic.—

Place 10 c.c. gastric juice in a casserole. Add 1 drop of Topfer's reagent. A bright red color develops if free hydrochloric acid is present.

Place N/10 sodium hydroxide in a buret. Add the sodium hydroxide from the buret drop by drop to the solution in the casserole until the red color is replaced by a canary yellow. Stir constantly during the addition of the sodium hydroxide.

Read from the buret the number of c.c. of sodium hydroxide used. Calculate the number of c.c. of sodium hydroxide required for 100 c.c. of gastric juice. This gives the degree of acidity.

Example.—

2 c.c. of N/10 sodium hydroxide were used for 10 c.c. gastric juice. Ten times 2 c.c., or 20 c.c., would be required for 100 c.c. gastric juice. The degree of free hydrochloric acid is, therefore, 20.

Normal free hydrochloric acid is 20 to 40 degrees.

To change the degree of acidity to percentage, multiply by 0.00365.

It is sometimes advantageous to determine the amount of hydrochloric acid deficiency in cases of achlorhydria. To do this test, place 10 c.c. gastric contents in a casserole, add 2 drops of Topfer's reagent, and titrate with N/10 hydrochloric acid until the first red indicative of free hydrochloric acid appears. Calculate the amount of N/10 hydrochloric acid required to neutralize 100 c.c. gastric contents; this figure represents the degree of hydrochloric acid deficiency. This is not a routine test, and is useful only in those cases in which hydrochloric acid therapy can be used to bring the hydrochloric acid deficiency back to normal.

Total Acidity

The total acidity comprises free hydrochloric acid, loosely combined hydrochloric acid, acid salts, and organic acids such as lactic acid, butyric acid, and amino acids.

Reagents.—**1% Alcoholic Phenolphthalein.—**

Phenolphthalein	1.0 gm.
95% Alcohol	100.0 c.c.

N/10 Sodium Hydroxide (page 25).

Technic.—

Place 5 c.c. unfiltered gastric contents in a casserole. Add 2 drops of 1% alcoholic phenolphthalein.

Have a buret filled with N/10 sodium hydroxide. Add the sodium hydroxide from the buret a drop at a time to the mixture in the casserole, stirring constantly, until the mixture remains a deep pink color. This is the end point. Read from the buret the number of c.c. of sodium hydroxide used. Calculate the number of c.c. of sodium hydroxide required for 100 c.c. gastric contents. This gives the degree of total acidity.

Example.—

If 3 c.c. N/10 sodium hydroxide were used to neutralize 5 c.c. of gastric contents, multiply 3 c.c. by 20 = 60 c.c., or the degree of total acidity in 100 c.c. of gastric contents. Normal degree of total acidity is between 40 and 60 to 70.

Combined Hydrochloric Acid

Reagents.—

N/10 Sodium Hydroxide (above).

1% Sodium Alizarine Sulphonate Solution.—

Sodium alizarine sulphonate	-----	1.0 gm.
Distilled water	-----	100.0 c.c.

Technic.—

Fill a buret with N/10 sodium hydroxide.

Place 10 c.c. unfiltered gastric contents in a casserole.

Add 3 drops of sodium alizarine sulphonate solution.

Add the sodium hydroxide from the buret to the solution in the casserole, a drop at a time, stirring constantly, until it becomes a permanent violet color. This is the end point. Read from the buret the number of c.c. of sodium hydroxide used. Calculate the amount required for 100 c.c. to obtain the degree of uncombined acids. Subtract this amount from the degree of total acidity obtained as above. This gives the degree of combined hydrochloric acid.

Example.—

Total acidity was 60 degrees. It required 4 c.c. N/10 sodium hydroxide to neutralize 10 c.c. of gastric contents.

4×10 gives 40 c.c. of N/10 sodium hydroxide required for 100 c.c. of gastric contents, or the degree of all acids except the combined hydrochloric acid.
 $60 - 40$ equals 20 degrees of combined hydrochloric acid.

Normal combined acidity is 10 to 15 degrees.

Lactic Acid

Lactic acid is not found normally in gastric juice. It may be present after ingesting certain foods; for example, sour milk or foods prepared with it. If emptying of the stomach is delayed, or if the secretion of hydrochloric acid is low, lactic acid may be formed from fermentation of carbohydrates by such lactic acid forming organisms as the Boas-Oppler bacillus. This is a common finding in advanced carcinoma of the stomach.

Reagents.—

1% Carbolic acid.—

Liquefied carbolic acid	-----	1.0 c.c.
Distilled water to make	-----	100.0 c.c.

10% Ferric chloride.—

Ferric chloride	-----	10.0 gm.
Distilled water to make	-----	100.0 c.c.

Technic.—

To 15 c.c. of 1% solution of carbolic acid in a test tube add 10% ferric chloride until an amethyst color is produced. Usually 1 or 2 drops is enough.

Add a few drops of unfiltered gastric contents.

A canary color indicates lactic acid.

Kelling's Method

Fill a test tube with distilled water.

Add 2 drops of 10% ferric chloride. Mix.

Divide the contents in the test tube into two parts.

In the first half, place 1 c.c. filtered gastric juice.

If lactic acid is present, a deep yellow color develops. Compare this color with the color of the control tube (distilled water plus ferric chloride).

Strauss' Method

Place 5 c.c. filtered gastric juice in a small separatory funnel.

Add 20 c.c. ether. Hold the stopper in tightly, and shake thoroughly.

Allow to stand in an upright position until the ether separates.

Allow the bottom fluid to run out. Retain the upper 5 c.c. of ether.

Add to this ether extract 20 c.c. distilled water and 2 drops of 10% ferric chloride solution.

Shake gently.

A slight green color indicates the presence of 0.05% lactic acid. An intense yellowish green color indicates the presence of 0.1% lactic acid.

Reagent.—**Uffelmann's Method**

Add 3 drops liquid carbolic acid to 3 drops of 10% ferric chloride. Dilute with distilled water until an amethyst color is produced.

Technic.—

To 50 c.c. ether, add 5 c.c. filtered gastric juice. Shake thoroughly for ten minutes.

Collect the ether by means of a separatory funnel, or a dropper pipette.

Evaporate to dryness. Add to the residue 5 c.c. distilled water.

Add the dissolved residue to Uffelmann's reagent.

A canary yellow color indicates the presence of lactic acid.

Fractional Method of Gastric Analysis

This method possesses the advantage of emptying and washing the stomach before any test meal is given. A second advantage is that specimens are withdrawn at frequent intervals; this tends to show gastric physiology step by step.

Technic.—

The patient presents himself in the morning without breakfast. He is instructed to eat or drink nothing after the previous evening meal.

At 9:00 A.M. a Rehfuß tube is placed in the stomach. The stomach is then aspirated with a syringe so as to remove all the contents.

After all the contents are removed, the stomach is washed with 100 c.c. of warm water and the washing is continued until clear fluid is aspirated.

The tube is removed and an Ewald test breakfast is now given. This must be thoroughly masticated.

The tube is now reinserted and at fifteen-minute intervals 5 c.c. of the stomach contents are aspirated. This should be carried out during two hours, at which time the stomach should be thoroughly empty. The tube is now removed.

Each of the 5 c.c. portions is examined and also the fluid taken from the fasting stomach. Examinations are made for total acidity, free hydrochloric acid, and lactic acid. In addition, observations should be made concerning the appearance of bile, blood, etc. The emptying time of the stomach is also computed by judging the disappearance of starch from the specimens, indicating that the starchy material of the test meal has all passed into the duodenum.

Normally, the free and the total acidity fall abruptly from a high figure in the case of the resting gastric juice, say 20, down to almost nothing at the end of the first fifteen minutes. From this time on, the free and total acidity rise until one and one-half hours have passed, and it is up to 40. After this time, the values fall until the end of the two hours. The free and total acidity figures are reduced, due to the regurgitation from the duodenum at this time. This is confirmed by the fact that although the acidity falls, the total chlorides increase, indicating that acid is still being secreted although it is being neutralized by the alkalized regurgitation.

The fractional test meal is important in differentiating ulcers of the duodenum, pyloric end of the stomach, and ulcer of the body of the stomach; it is also important in the determination of carcinoma of the stomach. The following data with the test are usual findings:

In chronic ulcer of the duodenum, the free hydrochloric acid in fifteen minutes drops from about 25° to practically none, and then rises from fifteen minutes on, so that in thirty minutes it is likely to be at 30° , in sixty minutes at 60° , in one hour and thirty minutes at 50° , in two hours at 55° . At the same time, the total acidity, beginning at 35° , drops in fifteen minutes to 15° , and then gradually rises; so that at the end of forty-five minutes it stands at 70° , in one hour at 75° , in one hour and fifteen minutes at 80° , dropping at the end of two hours to 70° . In ulcer of the pyloric end of the stomach, at the beginning of the test, the free hydrochloric acid, which is absent, begins to rise in thirty-minute intervals from 2° , at forty-five minutes 10° , at one hour 20° , at one hour and fifteen minutes 35° , one hour and thirty minutes 35° , one hour and forty-five minutes 40° and at two hours 50° . The total acidity, in the meantime, beginning at 10° , drops at the end of fifteen minutes to 5° , and then gradually rises in thirty minutes to 20° , and forty-five minutes to 30° , one hour to 40° , and one hour and fifteen minutes to 70° , one hour and thirty minutes to 75° , and then gradually declines until at the end of two hours it is at 60° . In ulcer of the fundus of the stomach, we find no free hydrochloric acid in fifteen minutes, none in thirty minutes, in forty-five minutes 10° , in one hour 25° , one hour thirty minutes 40° , when it gradually drops so that in one hour and thirty minutes it is 25° , and in two hours 20° . In the meantime, the total acidity, beginning at 15° , drops in the first fifteen minutes to 10° and gradually rises, at thirty minutes to 20° , forty-five minutes to 25° , one hour to 40° , one hour and fifteen minutes to 55° , when it remains stationary, and then gradually drops at the end of one hour and forty-five minutes to 40° , and in two hours to 35° .

In carcinoma of the stomach, there is no free hydrochloric acid at any time. The total acidity, beginning at 10° , drops in fifteen minutes to 5° , then gradually rises in thirty minutes to 10° , in forty-five minutes to 12° , in one hour to 15° , in one hour and fifteen minutes to 20° , when it gradually drops so that at the end of two hours it is again at 10° . In all the specimens, blood and mucus are likely to be found.

In pernicious anemia, no free hydrochloric acid is found in any of the specimens. The total acidity begins at about 8° and runs on almost a straight line of 8° to 10° throughout all the specimens. Bile and mucus are likely to be found in all the specimens.



PLATE XXIX.—TITRATION OF STOMACH CONTENTS.

1. Contents With Topfer's Reagent Showing Presence of Free Hydrochloric Acid.
2. End Point in Titration for Free Hydrochloric Acid.
3. End Point in Titration for Total Acidity.
4. End Point in Titration for Combined Hydrochloric Acid.



Acidity in Various Conditions

Achlorhydria.—The continued absence of free hydrochloric acid in the stomach contents is not in itself a disease entity. There are very few diseases characterized by achlorhydria. In these cases additional clinical and laboratory findings will aid in the proper diagnosis. The lack of titratable acidity in some patients is due to bile and duodenal regurgitation which neutralizes the acid output of the stomach. This kind of achlorhydria can be determined by the estimation of chlorides and trypsin in the gastric contents. While no free acid may appear in the first hour, the study of an acid curve which appears after sixty minutes may be helpful. Achlorhydria is found in the following diseases:

1. **Pernicious Anemia.**—There is very seldom any free hydrochloric acid present in pernicious anemia. The ferments, rennin and pepsin, are also absent. The absence of free hydrochloric acid and of the ferments in the gastric contents may be found long before the blood picture becomes characteristic. Even though liver therapy or vitamin B₁₂ may bring about a normal blood count, yet the achlorhydria remains.

2. **Subacute Combined Sclerosis** resembles pernicious anemia. Every person with achlorhydria is a potential case either of subacute combined sclerosis of the spinal cord or of pernicious anemia. Subacute combined sclerosis is symptomatic of pernicious anemia, but may occur in the course of a leukemia.

3. **Sprue**, which resembles pernicious anemia, is always accompanied by achlorhydria.

4. **Gall Bladder Diseases.**—A diseased gall bladder that has lost its function is likely to be accompanied by an absence of or by a great decrease in free hydrochloric acid in the stomach contents. Wohl¹ particularly calls attention to this fact. Others have corroborated his findings, the particular significance of which lies in the following fact: In a patient with symptoms in the upper abdomen, in whom gastric carcinoma has been ruled out and in whose gastric contents no free hydrochloric acid has been found, the gall bladder should be strongly suspected as the etiological factor responsible for the symptoms.

5. **Diabetes Mellitus.**—In certain cases of this disease with lack of appetite, there is absence of or great decrease in free hydrochloric acid. Their symptoms improved when these patients were given dilute hydrochloric acid.

6. **Chronic Gastritis.**—In this condition the acidity may vary from time to time. Gastritis of the fundus tends toward the absence of hydrochloric acid.

7. **Carcinoma of the Stomach.**—Absence of free hydrochloric acid, lactic acid in the gastric contents, occult blood in the stool, defect and slow emptying of the stomach in x-rays, all favor the diagnosis of malignancy. (See remarks on diagnosis of gastric carcinoma by Papanicolaou and Cooper, method of balloon diagnosis of gastric contents, pages 2193 ff.)

8. **Pellagra.**—Achlorhydria is often encountered in this disease:

¹Wohl, M. G.: *Bedside Interpretation of Laboratory Findings*, The C. V. Mosby Co., St. Louis, 1931.

9. **Syphilis.**—According to Marcus, there is always present a gastric sub-acidity, anacidity, or complete achlorhydria in this disease.

10. **Amebic Dysentery.**—Porgess states that in amebic dysentery the gastric contents frequently show achlorhydria.

11. **Achylia Gastrica.**—Here free hydrochloric acid is absent, and the total acidity rarely rises above 10. The gastric enzymes are absent, due to atrophy of the mucosa. No gastric secretion follows the injection of histamine. Normally 0.1 mg. of histamine per 10 kilograms body weight, injected hypodermically, will stimulate gastric secretion.

12. **Miscellaneous Group.**—Hypothyroidism, rheumatoid arthritis, and pulmonary tuberculosis may frequently be associated with achlorhydria.

Hyperacidity

Hyperacidity is a common occurrence. It is found in patients with neuroses, in cases of constipation, and in excessive cigaret smokers. The eating of too many pickles, or of foods rich in condiments, or of excessive amounts of carbohydrates may produce it.

Gastric and duodenal ulcers are frequently found in association with high acidity; however, in about 50 per cent of gastric ulcer cases, the acidity is normal. In duodenal ulcer the high acidity factor is more constant. The point of high acidity is usually reached in from forty-five minutes to two hours after eating the test meal. The degree of gastric acidity helps to determine the degree of activity of the ulcer; thus, when there is a low total acidity, the ulcer is chronic, inactive, as a rule much indurated, or has gone over to the carcinoma phase.

Occult Blood

Benzidine Test.—Prepare about 3 c.c. of a saturated solution of benzidine (special for blood) in glacial acetic acid in a test tube.

Add an equal volume of 3% hydrogen peroxide. Mix thoroughly. Should the solution turn green or blue, it must be discarded.

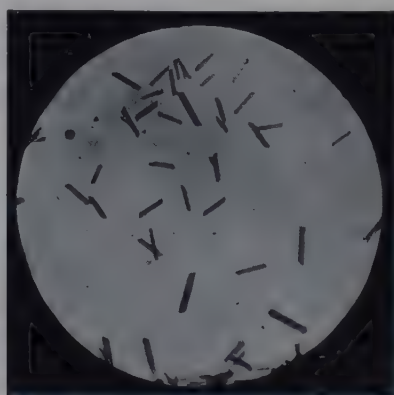


Fig. 292.—Hemin crystals. ($\times 100$.)

Add a few drops of unfiltered gastric contents and shake.

A green or blue color indicates the presence of occult blood. (See pages 1289 ff.)

Teichmann Test for Hemin Crystals.—

Disintegrated blood must be examined chemically by the hemin test of Teichmann. Mix a small amount of the suspected material with a crystal or two of common salt, or place it on the thin layer of salt formed by slowly evaporating a small drop of physiologic salt solution on a slide. Cover with a cover glass, and run in enough glacial acetic acid to fill up the

space between slide and cover. Warm the slide over a flame for three-quarters to one minute until bubbles arise, adding more glacial acetic acid until a faint reddish brown tint appears. Then let the acetic acid evaporate entirely, and run distilled water in from the edge of the cover glass.

Microscopic examination will show dark brown rhombic plates or columns of hemin if blood is present.

Benzidine Dihydrochloride Test.—Follow the method given on page 84, substituting gastric contents for urine.

Benzidine Test, Method of Levin and Watt¹.—Mix the gastric or duodenal contents thoroughly with an equal volume of distilled water.

Filter through 1 or 2 Whatman No. 5 extra-retentive filter papers to obtain a clear filtrate.

To 3 c.c. of the clear filtrate, add 5 drops of 50% acetic acid and 5 drops of hydrogen peroxide, and shake vigorously.

Overlay the mixture with 0.5 c.c. of 6% alcoholic benzidine solution and rock slightly. The benzidine solution should preferably be 2 or 3 days old.

A positive reaction is indicated by a green ring at the junction of the benzidine solution and the filtrate mixture.

This test can be used for detecting **blood in vaginal washings**. Sterile water irrigation of the posterior portion of the vagina is performed with an Asepto syringe of 10 to 20 c.c. capacity.

Perform the test without filtration, and, if positive, follow by Papanicolaou examination. The test is positive in cases where there are 4 to 6 red blood cells per high-power field.

If urine is to be tested, centrifuge 10 c.c. at 1,500 r.p.m. for 3 minutes, decant the supernatant fluid, and use the sediment.

Add 1 c.c. of water and 3 drops of 50% acetic acid, shake, and add 3 drops of hydrogen peroxide. Shake vigorously.

Overlay the mixture with 0.5 c.c. of 6% alcoholic benzidine solution, and observe for a green ring at the point of contact of the liquids.

If feces are used, emulsify a small portion in distilled water, filter as for gastric contents, and use 3 c.c. of the filtrate, 8 drops of the acetic acid, and 8 drops of hydrogen peroxide before overlaying with the alcoholic benzidine solution.

Bile

Break up a match stick in about 5 c.c. nitric acid (concentrated). Stratify with a few c.c. of gastric juice.

A play of colors will develop at the point of contact if bile is present.

Butyric Acid

Butyric acid is manifested by the rancid, butter-like odor. If a test is desired, boil the gastric contents to accentuate this odor.

If a piece of moistened blue litmus paper is held in the mouth of the tube, the volatile acid reddens it as it escapes during the boiling. Butyric acid also separates as a drop of oil if a piece of calcium chloride is added to it.

Rennin-Renninogen

Rennin is the milk-curdling ferment of the gastric juice. It is derived from renninogen through the action of hydrochloric acid. Deficiency of rennin has the same significance as deficiency of pepsin, and is more easily recognized.

¹Rev. Gastroenterol. 16: 650, 1949.

Test: Neutralize 5 c.c. of filtered gastric juice with very dilute sodium hydroxide solution. Add 5 c.c. of fresh milk, and place in an incubator or in a vessel of water at about 40° C.

Coagulation of the milk in ten to fifteen minutes shows a normal amount of rennin. Delayed coagulation denotes a less amount.

Pepsin*

“**Tests for Identity.**—An aqueous solution of pepsin (1 in 50) is acid to litmus paper. An aqueous solution of pepsin yields precipitates with solutions of tannic or gallic acid and with solution of the salts of many heavy metals.

“On heating a saturated solution of Pepsin in acidulated water to 100° C., it becomes milky or yields a light, flocculent precipitate, and loses all proteolytic power.

“**Technic.**—Mix 25 c.c. of normal hydrochloric acid with 275 c.c. of distilled water. Dissolve 0.1 gm. of Pepsin in 150 c.c. of this acid. Select a hen’s egg, which is not less than 5 and not more than 12 days old and has been kept in a cool place, and immerse it in boiling water during 15 minutes. As soon as the egg has sufficiently cooled to handle, remove the shell and pellicle and all of the yolk and at once rub the albumen through a clean, dry, hair or brass, No. 40 sieve, reject the first portion that passes through the sieve, and place 10 gm. of the succeeding portion in a wide-mouthed bottle of 100 c.c. capacity. Immediately add 20 c.c. of the acid and stir the mixture well with a rubber-tipped glass rod so as to separate thoroughly the particles of albumen from each other. Rinse the rod with 15 c.c. more of the acid and, after warming the mixture to 52° C., add exactly 5 c.c. of the acidulated solution of Pepsin. At once cork the bottle securely, invert it 3 times, and place it in a bath that has been previously regulated to maintain a temperature of 52° C. Keep the bottle at this temperature for 2½ hours, agitating the contents every ten minutes by inverting it once. Then remove it from the bath, pour the contents into a conical measure having a diameter not exceeding 1 cm. at the bottom, and transfer the undigested egg albumen, which adheres to the sides of the bottle, to the measure with the aid of small portions (about 15 c.c. at a time) of distilled water, until the total amount used measures 50 c.c. Stir the mixture well and allow it to stand for half an hour. The deposit of undissolved albumen does not measure more than 1 c.c.

“The relative proteolytic power of Pepsin, stronger or weaker than that just described, may be determined by ascertaining through repeated trials the quantity of the Pepsin solution made as directed in the assay, required to digest, under the prescribed conditions, 10 gm. of boiled and disintegrated egg albumen. Divide 15,000 by this quantity expressed in c.c. to ascertain how many parts of egg albumen one part of Pepsin will digest.

“Preserve in well-closed containers.”

MICROSCOPIC EXAMINATION

To make a microscopic examination, place a drop of unfiltered stomach contents upon a slide, cover with a cover glass, and examine with a low dry and a high dry objective with the diaphragm opening much reduced. If a drop of diluted Lugol’s solution is placed on the edge of the cover glass, it will run under the cover by capillary attraction and will help to bring out certain structures. (See pages 1627 ff. and 1609 ff. for examination of gastric contents for tubercle bacilli.)

Normally, we see starch granules, with an occasional epithelial cell, yeast cell, or bacterium. Starch stains blue with iodine solution when undigested,

*Reprinted by permission from The Pharmacopoeia of the U. S. A., X.

and reddish, due to erythrodextrin, when partially digested. These colors, however, are not shown clearly unless the iodine solution is very dilute.

Under pathologic conditions, we see remnants of food from previous meals, red blood corpuscles, pus cells, sarcinae, and excessive numbers of yeast cells and bacteria.

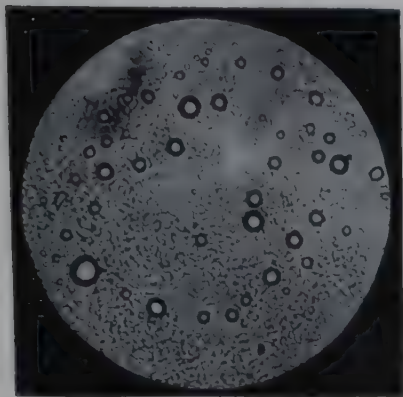


Fig. 293.

Fig. 293.—Fat globules. ($\times 400$.)



Fig. 294.

Fig. 294.—Starch granules. Squamous epithelial cells.

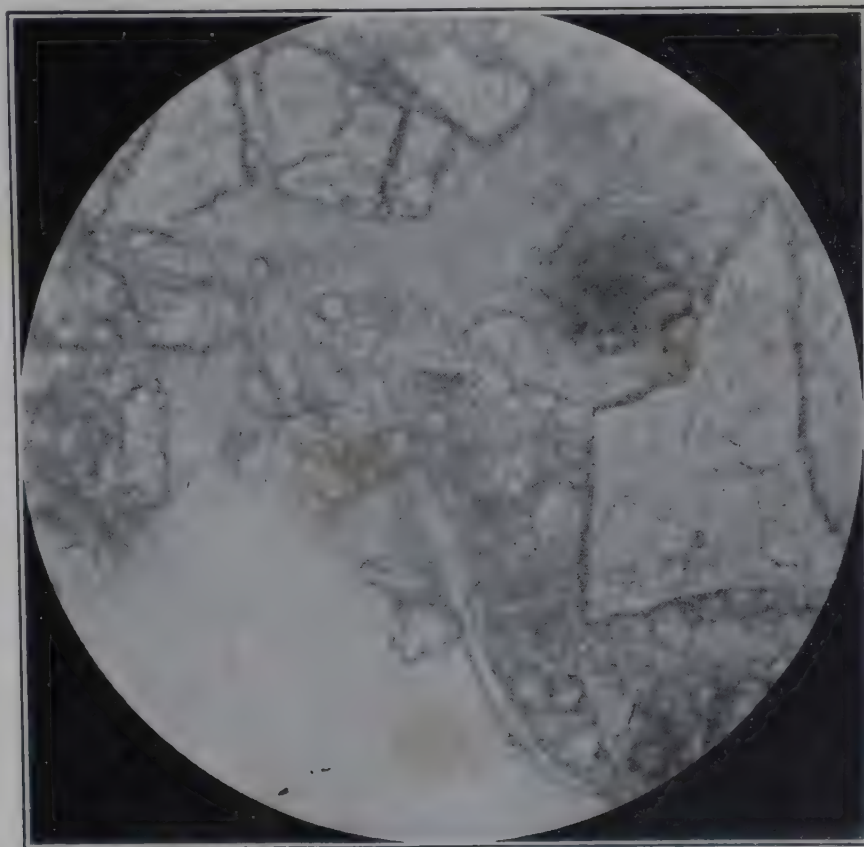


Fig. 295.—Cholesterol and carbonate crystals; slight amount of calcium bilirubinate pigment. (Photomicrograph of particle in preoperative specimen of gastric lavage.) (From Rafsky: *J. Lab. & Clin. Med.*, June, 1934.)

Remnants of food from previous meals indicate deficient gastric motility. Red blood cells are recognized by chemical tests, preferably the *benzidine* test. Microscopically, the red blood corpuscles retain a fairly normal appearance but are so degenerated at times that only granular pigment is left. The presence of very fresh-looking red corpuscles is highly suggestive of irritation

of the stomach mucosa by the stomach tube. Pus cells are rarely seen in gastric contents although many of them have been seen in cases of gastric cancer. These corpuscles are partially digested, only the nuclei remaining. The nuclei appear as highly refractile bodies.

Swallowed sputum may be present. See pages 1609 ff. and 1627 ff. for examination of gastric contents for tubercle bacilli.

Sarcinae are small spheres arranged in cuboid groups, often compared to bales of cotton. They are easily recognized and stain brown with iodine solution. They are rarely found in cases of gastric carcinoma. A few yeast cells may be found under normal conditions with considerable numbers in retention and fermentation. They stain yellow to brown with iodine solution. The bacteria encountered are not very important except the Boas-Oppler bacillus. It is claimed that this is found in the majority of cases of carcinoma of the stomach and rarely in any other condition. This organism belongs to the *Lactobacillus bulgaricus* type. The Boas-Oppler bacillus is large, 5 to 10 microns long, nonmotile, and arranged in clumps or in zigzag chains. It stains yellow to brown with iodine solution, thus distinguishing it from *Leptotrichia buccalis* which is sometimes swallowed and may be found in stomach fluid.

Crystalline Elements in Stomach Lavage.—Rafsky¹ reported that crystalline elements were present in the stomach lavage of patients with cholelithiasis. In an effort to approximate this finding, this subject was further investigated in a series of patients with and without biliary tract disease. This study was aided by the use of photomicrographs of crystals seen in the stomach lavage before operation, which were compared with photomicrographs of crystals found in the bile and stones removed from the gall bladder and bile ducts at the time of operation.

Seventy-nine patients were observed and grouped as follows:

Group I. Forty-one patients (51.9 per cent) in whom biliary tract disease was suspected from the history, but who were not operated upon.

Group II. Eighteen patients (20.3 per cent) in whom biliary tract disease was suspected from the history, but who were not operated upon.

Group III. Twenty patients (27.8 per cent) who comprised the control group. In these patients there was no history of biliary tract disease.

The method of procedure was as follows: A gastric lavage with warm tap water was performed upon each patient, the lavage being given preferably in the morning, while the patient was still fasting. The lavage water was then collected and examined for crystalline elements. When crystals were present they were usually found in the greyish or brownish red particles or in the mucus.

In the patients in Group I and Group II, the gastric lavage was made in the morning following an attack of biliary colic or gastric disturbances of sufficient severity to disturb the patient's sleep. When the lavage water was obtained under these circumstances, crystalline elements were found more consistently and in larger amounts.

¹Rafsky, H. A.: J. Lab. & Clin. Med. 19: 959, 1934.

CONCLUSIONS TO BE DRAWN FROM THE EXAMINATION OF GASTRIC CONTENTS

A number of important points and diagnoses are deducible from examination of the gastric contents. First, the emptying time of the stomach is in a measure determined by this examination. If there is considerable fluid or food particles in the gastric contents, it is reasonable to assume that the case is one of pyloric obstruction, probably caused by a malignant condition of the pylorus or some formation with contraction due to a chronic gastric ulcer. The presence of blood in the gastric contents in visible amount is necessarily due to hemorrhage from an ulcer or carcinoma. Due consideration, of course, must be given to the possibility of a small amount of blood sometimes caused by the passage of the stomach tube.

If there is no free hydrochloric acid present after a test meal, we are inclined to think of the following conditions—pernicious anemia, primary hypochromic anemia, and cancer of the stomach. In pernicious anemia there is complete absence of hydrochloric acid, whereas in cancer a small amount may be present at times, although this is rare. In certain persons, with neither cancer nor pernicious anemia, the nervous excitement caused by the passage of the tube may probably suppress the secretion of hydrochloric acid. In such cases the injection of a preliminary drop of histamine will override this suppression due to nervousness and one can obtain an idea of the true condition. If, however, after histamine injection, no free hydrochloric acid is found, this is necessarily a true achlorhydria.

When increased hydrochloric acid is seen in those persons having ulcers, it is due to hypersecretion by the parietal cells of the stomach. That there is a relationship between hydrochloric acid and peptic ulcer is evidenced by the fact that those with histamine achlorhydria virtually never develop either duodenal or gastric ulcers.

In *dilatation of the stomach*, there is evidence of retention and fermentation, with hydrochloric acid diminished. Pepsin may be normal or slightly diminished. Lactic acid may be detected in small amounts, but is usually absent when the stomach has been washed before giving the test meal. Microscopically, we find sarcinae and other bacteria, and great numbers of yeast cells.

In *gastric neuroses*, the findings are variable. There may be normal, increased, or diminished hydrochloric acid, or even entire absence of the free acid. Pepsin is usually normal.

In *chronic gastritis*, free hydrochloric acid may be increased in early cases, but it is usually diminished in well-marked cases, and is often absent in advanced cases. Lactic acid may be present in traces. Pepsin and rennin are diminished in marked cases. The presence of large amounts of mucus is very characteristic of this condition.

In *atrophic gastritis*, or *achylia gastrica*, which is the terminal stage of chronic gastritis, associated with the blood picture of pernicious anemia, there is decrease in, with sometimes entire absence of, free hydrochloric acid and ferments. Total acidity may be down to one degree. Small amounts of lactic acid may be present.

Gastric carcinoma is possibly the most important condition which we have in mind in examination of gastric contents. There is a total absence of free hydrochloric acid and the presence of lactic acid and of the Boas-Oppler bacillus. Exceptions to this have been found where some free hydrochloric acid and no lactic acid have been found in undoubted cases of carcinoma of the stomach. It must be borne in mind, however, that the presence of lactic acid is very strong presumptive evidence of the existence of carcinoma. In gastric carcinoma, we often find blood, especially when the tumor is located at the pylorus. In these cases occult blood is also likely to be found in the stool.

Gastric ulcer is associated with an excess of free hydrochloric acid in about one-half of the cases. In other cases the acid is normal or diminished. The presence of blood is a very significant symptom of ulcer. It is especially important in these conditions to make an examination of the stool for occult blood, taking care, of course, to have the patient on a meat-free diet for four or five days before making the test.

Summary.—Examination of the gastric contents is of greatest value in the diagnosis of gastric ulcer, gastric cancer, and severe anemia of both pernicious and primary hypochromic type. In gastric ulcer, blood, an excess of hydrochloric acid, and perhaps gastric retention are found. In cancer there is very little or no hydrochloric acid, the presence of blood, together with gastric retention and lactic acid if the growth is near the pylorus. Finally, in pernicious anemia and primary hypochromic anemia there will be a complete absence of hydrochloric acid, no blood, no gastric retention or lactic acid.

GASTROSCOPY AS AN AID TO GASTRIC DIAGNOSIS

The following facts were well presented by Deutsch.¹ Gastroscopic study is indicated (1) in x-ray negative dyspepsia, (2) to clarify an indefinite roentgenographic impression or confirm a positive diagnosis of carcinoma, and (3) in all patients with gastric ulcer or polyps.

Gastroscopy can help in distinguishing benign from malignant ulceration of the stomach. In the antrum and middle portions of the stomach, observation of peristaltic waves and segmental tonus changes aids in ruling out an infiltrating rigid neoplastic lesion. Infiltrating lesions in the cardia or fundus show evidence of obstruction at gastroscopy as much as six months before it is detected at x-ray.

Again, positive biopsies have been taken during gastroscopy. Exfoliative cytology, accomplished by scraping with a polyethylene tube under gastroscopic observation, yields a desquamated mucosa and muscularis mucosa which is quickly aspirated, fixed in Formalin, and prepared for cell block.

Attention is here called to the balloon technic of Cooper, described on page 2193 on the exfoliative diagnosis of gastric lesions.

Radiology and gastroscopy are complimentary aids in the diagnosis of diseases of the stomach. It must be remembered that gastric secretion or mucosal folds in tetanic spasm in a very obese patient occur frequently and make mucosal relief studies by x-ray difficult. At gastroscopy these factors are no obstacle to an adequate examination.

¹Deutsch, Emmanuel: *Postgrad. Med.* 16: 413, 1954.

PHYSIOLOGIC DATA ON GASTRIC SECRETION

In addition to the purely chemical tests already outlined, there are a few tests based upon physiologic reactions which are important in diagnosis.

Determination of the Absorptive Power of the Stomach

Conclusions from this test must be drawn very carefully, otherwise errors will be made. All other factors in connection must be considered together with the facts brought out by this test. It is commonly believed that in organic diseases of the stomach, such as carcinoma and gastrectosis, the absorptive power of the stomach is delayed, but in the neuroses, it is not delayed.

Method: The patient, on an empty stomach, takes a 3-grain capsule of potassium iodide and a glass of water. Be sure that the entire drug is taken from the inside of the capsule. From time to time, the saliva is tested for iodides by moistening starch-paper with it and touching with yellow nitric acid. A blue color shows the presence of iodides. Normally, this should appear in about ten to twenty-five minutes after the ingestion of the drug. If it does not appear until after thirty minutes, it may be assumed that the absorptive power of the stomach is delayed. In order to facilitate the performance of the test, prepare starch-paper by soaking filter-paper in boiled starch and drying.

Test for Motor Power of the Stomach

This test is intended to determine how rapidly the stomach contents pass into the intestine. It is an important aid to diagnosis for the reason that intestinal digestion may compensate for insufficient or absent stomach digestion only so long as the motor power of the stomach is good. Gastric motility is imperative. It is deficient in the following conditions: in pyloric obstruction caused by malignant or benign new growths; in pyloric spasm, as in hyperchlorhydria; and in atony of the stomach wall. It is usually associated with gastrectosis and gastroptosis. To detect deficient motor power, we attempt to find food in the stomach before breakfast in the morning; that is, at a time when the stomach should be empty. Give the patient a meal containing such materials as rice puddings with currants or jam with seeds or raisins, and remove it at the end of six or seven hours. If more than 100 c.c. of fluid can be removed with a stomach tube one hour after an Ewald breakfast, it is an indication of deficient motility of the stomach.

EXAMINATION OF THE DUODENAL CONTENTS

Examination of the duodenal contents is primarily indicated to determine whether or not there is an impaction of the bile duct by gallstones. It indicates the presence or absence of inflammation and stones in the gall bladder. This absence of bile in duodenal contents may be due to a gallstone impacted in the duct or to a cancer at the head of the pancreas. If the bile contains pus cells, it is strongly suggestive of acute inflammation of the gall bladder. Crystals of cholesterol and granules of bile pigment may be found. If both of these are present in considerable amounts, it is presumptive evidence that there are stones in the gall bladder.

Method of Withdrawal

The duodenal fluid is obtained by means of a slender, flexible rubber tube, from 3 to 4 mm. in outside diameter, with a perforated metal tip. It is marked with a series of black rings to indicate the distances from the incisor teeth to the cardia, to the pylorus, and to the duodenum. The Rehfuß tube is the instrument of choice.

If the pancreatic ferments are to be studied, give a cup of bouillon a half-hour before the tube is introduced. In most of the duodenal contents examinations, however, a test meal is not used. The patient abstains from food for about twelve hours, taking only occasional sips of water. The tube is inserted into the stomach in the manner described for the stomach tube. Place the patient upon his right side with hips elevated 6 or 8 inches. The movements of the stomach, aided by gravity, carry the metal tip through the pylorus into the duodenum, usually within thirty to forty-five minutes. Fluid begins to drip from the free end of the tube soon after the metal tip has reached the stomach. If it does not do so, siphonage should be started by injecting a few cubic centimeters of warm water. Collect the fluid in portions of about 5 or 10 c.c. in a series of test tubes. The fluid which first appears is from the stomach and may be recognized by its acid reaction. When the tube enters the duodenum, the fluid becomes slightly alkaline and is usually clear, light yellow or colorless, and viscid. If one is in doubt as to whether or not the tube is still in the stomach, give the patient a few swallows of milk, and aspirate a portion of the fluid through the tube. If the milk appears, the tube is still in the stomach. If the flow should at any time be interrupted, inject a few c.c. of warm water. If, after the tube is in the duodenum, the fluid is somewhat cloudy and opalescent, less alkaline and less viscid, this is the result of admixture with stomach fluid which may have passed through the pylorus. Such fluid is to be discarded.

Examination of duodenal fluid should be done as soon after withdrawal as possible since many of its characteristics quickly change. Normally, it is clear, colorless or light yellow, distinctly viscid, and slightly alkaline. Admixture of acid gastric juice causes it to become somewhat cloudy and opalescent. Cloudiness due to bacteria and pus corpuscles may be present in inflammation of the duodenum or biliary passages.

Lyon-Meltzer Method of Duodenal Drainage*

Drainage of the gall bladder by inducing a relaxation of the duodenum, through the introduction into it of a 25 per cent solution of magnesium sulphate, has attracted a great deal of attention. Lyon claims that by adhering closely to his technic many cases of early gall tract disease can be diagnosed from the examination of the duodenal contents. It has been proved, however, that the so-called A-, B-, and C- bile, presumably originating from three different parts of the biliary tract, have no clinical significance. (The A- bile is supposed to originate in the common duct; the B- bile, a dark viscous bile, in the gall bladder, and the C- bile, a golden yellow bile, in the hepatic duct.) Lintz reported a case that has dealt quite a blow to the A-, B-, and C- bile contention. His patient had congenital absence of the gall bladder and yet,

*Wohl: *Bedside Interpretation of Laboratory Findings*, St. Louis, The C. V. Mosby Co.

upon injection of magnesium sulphate, the three typical specimens of bile, including the dark B- bile, which is supposed to come from the gall bladder, appeared. Nevertheless, a careful microscopic examination of duodenal sediments may be of diagnostic value.

According to Jones, bile-stained leukocytes and epithelial cells, when found in any quantity, indicate an inflammatory condition of the biliary tract. It is not possible, though, to determine from the type of cell found in the duodenal sediments the exact level of the inflammatory process. Too much importance should not be attributed to the finding of these cells; they should be regarded merely as confirmatory evidence.

The presence of many crystals of cholesterin, bilirubin, and calcium bilirubinate may be of diagnostic value in cholelithiasis. It has been claimed by Bockus and his associates that the finding of cholesterol crystals and bilirubin calcium pigment in the same bile is pathognomonic of cholelithiasis.

Chemical Examination of Duodenal Contents

(A) Ferments

Estimation of the pancreatic ferments, amylapsin, steapsin, and trypsin, in duodenal contents, gives the same information as their estimation in feces, but is more reliable. Absence of or great decrease in the quantity of one or all of these ferments points to deficiency of the pancreatic secretion or occlusion of the pancreatic duct. Pancreatic fluid from a fistulous tract shows little or no proteolytic activity. The following methods, although not absolutely accurate, give important clinical information.

1. Amylopsin (Pancreatic Amylase) should be studied along with amylase of urine and blood (pages 140, 141, 374 ff.). Carry out the technic for amylase in feces, but use pure duodenal fluid, 5 c.c. of 1% starch, and bring the volume in each tube to 7 c.c. Express the amount of amylase in units (the number of c.c. of 1% starch digested by 1 c.c. of duodenal contents). For example, if the digestion of starch is complete in the tube containing 0.2 c.c. of duodenal fluid, 25 units of amylase are present. According to Myers and Fine, the average is about 40 units, with extremes of 5 and 200 units in different specimens.

2. Steapsin (Pancreatic Lipase) (Myers and Fine).

Place 1 c.c. duodenal fluid in each of two test tubes.

Boil the contents of one tube to destroy the ferments.

Add to each tube 1 c.c. neutral ethylbutyrate, 10 c.c. distilled water, and a few drops of toluol.

Incubate at 38° to 40° C. for twenty-four hours, shaking occasionally. If lipase is present, the ethylbutyrate will be split, the resulting fatty acid giving an acid reaction in the tube which was not boiled.

Add 2 drops phenolphthalein indicator to each.

Titrate to a pink color with N/20 sodium hydroxide.

The index of lipolytic activity is the difference in the number of c.c. of N/20 sodium hydroxide required for the two tubes. According to Myers and Fine, this is 0.3 to 4.3 c.c., averaging 1.5 to 2.

3. Trypsin.—

If the fluid is acid or neutral, render it slightly alkaline with a weak solution of sodium carbonate.

To 5 c.c. of the fluid in a test tube, add 1 gram of fibrin and a few drops of toluol.

Incubate at 38° to 40° C. for twenty-four to forty-eight hours. Tyrosin and tryptophan can be found if trypsin is present. Neutralize the fluid and examine microscopically for

tyrosin crystals. Test for tryptophan by adding very dilute bromin water a drop at a time. If tryptophan is present, a reddish violet color develops, which disappears upon the further addition of bromin.

4. Amylase.—

To 10 c.c. of 1% solution of freshly prepared starch
add 1 c.c. of the pancreatic juice
and 1 c.c. of toluol.

Allow the mixture to digest at 40° C. until duplicates no longer show blue with iodine solution. Place the bottles in boiling water to stop digestion at the same moment.

Dilute the contents to 50 c.c.

Titrate from a buret against 10 c.c. of boiling Fehling's solution, using acetic acid and potassium ferrocyanide to determine the end point.

Ascertain the number of c.c. of digestive mixture required to reduce 10 c.c. of Fehling's solution, which is equivalent to 0.05 gm. of dextrose, or its equivalent of maltose.

If the amount of sugar in the digestive mixture is less than that required to reduce the copper, add a standard dextrose solution, and compute the amount of dextrose equivalent in the juice used.

Amylase values are reported as the number of c.c. of the digestive mixture, which, when made up to 50 c.c., would be equivalent to 0.05 gm. of glucose. Normal values are given above.

5. Lipase.—

Place in a bottle 1 c.c. of the pancreatic juice.

Add 10 c.c. distilled water

and 1 c.c. of neutral ethyl butyrate

and 1 c.c. toluol

and allow to digest at 40° C.

After 24 hours, determine the amount of acidity developed, using N/20 sodium hydroxide aqueous solution, with phenolphthalein indicator. From the amount of sodium hydroxide used subtract the amount of acidity, if any, in the duplicates containing 1 c.c. of juice plus 10 c.c. water and 1 c.c. toluol.

Shake the digestive mixtures ten times during the 24-hour period.

Express the lipase values as the amount of N/20 sodium hydroxide necessary to neutralize the acidity developed. Normal values are given on page 1213.

6. Alkali-protease (Trypsin).—

Dissolve 10 gm. gelatin

in 100 c.c. of 1% solution of sodium fluoride

and color with methyl violet.

Draw this solution into capillary tubing 1 mm. in diameter.

Quickly place the tubing under cold water to solidify the gelatin.

Cut the tubing into 2 cm. lengths.

Place 10 c.c. of the juice in a small bottle.

Close with a perforated cork through which the gelatin tubes can be inserted.

Keep the digestions at room temperature for 48 hours to determine the alkali-protease.

Add 1 c.c. of toluol to each digestive mixture

and enough 0.2% sodium carbonate solution to make the mixture alkaline in reaction.

Express the alkali-protease (trypsin) values in the number of millimeters of gelatin digested. Normal value is 25 to 50.

Quantitative Methods of Determining the Pancreatic Ferments (Einhorn¹)

Principle.—Agar tubes, mixed with albumin, starch, or fat, allow the ferment action to take place in them by osmosis. If the test substances added are colored

¹From Einhorn, Max: *The Duodenal Tube and Its Possibilities*, ed. 2, 1926, F. A. Davis Company, p. 31, by permission of Dr. Einhorn and the publisher.

with indicators, which undergo a change when acted upon, it is easy to ascertain the presence of the ferments, and also to estimate their approximate amount by the volume of tube changes given in millimeters.

Starch Tubes.—

Agar powder -----2.5 gm.
 Starch -----5.0 c.c.
 Distilled water up to 100 c.c.

Rub the starch and agar in a mortar with sufficient water to make a smooth paste, then add the rest of the water. Place in a flask and heat to boiling point. Draw by suction into a capillary glass tube, inside diameter 1.5 mm., which has previously been warmed in the flame. Allow tube and contents to cool. Cut into 2 cm. lengths. Seal at each end with melted paraffin.

Olive Oil Tubes.—

Olive oil -----25 c.c.
 Agar powder ----- 2 gm.
 Aqueous solution of Nile-blue sulphate (1:2000),
 sufficient to make 100 c.c.

Rub the olive oil and agar together. Add sufficient water to make a thin paste. Add the Nile-blue sulphate solution to make the balance of 100 c.c. Then proceed as with the starch tubes.

Hemoglobin Tubes.—

Hemoglobin powder -----1.0 gm.
 Agar powder -----2.5 gm.
 Distilled water sufficient to make 100 c.c.

Rub the hemoglobin with about 10 c.c. of water until it is smooth, then add the agar powder and the balance of the water. Proceed as with the starch tubes.

Keep the agar tubes on ice until ready to use. They retain their efficiency for a month or six weeks, when they begin to deteriorate; they should not be used when they become dry.

Technic.—

Use one each of the starch, oil, and hemoglobin tubes.

Scrape off the paraffin at both ends.

Put vertically into a small bottle containing about 0.5 c.c. of duodenal secretion.

Add 1 to 2 drops of toluol and keep the bottle at blood temperature for from sixteen to twenty-four hours.

Examine as follows:

Take the agar tubes from the bottle, wipe the outside, and inspect. Hemoglobin tubes show a change of appearance due to action of trypsin, with a clearing up of the end part, becoming more or less transparent. Oil tubes manifest bluish appearance at the end because steapsin ferment splits the oil into fatty acids, which produce this color. Measure the lengths of transparency in the hemoglobin tube and of the bluish color in the oil tube in millimeters. Examine the starch tube by pushing out the agar column and dipping it into a weak iodine solution. The portion of starch column remaining colorless indicates the part changed by the amylopsin ferment into sugar. This is expressed in millimeter lengths.

By these tests, the hemoglobin serves to gauge the trypsin, the olive oil, the steapsin, and the starch the amylopsin ferments.

Fractional Examination of the Duodenal Contents.¹—Einhorn recommends a test meal of one cup of beef broth, usually made by putting one bouillon cube

¹Einhorn, Max: Am. J. M. Sc. 156: 817, 1918.

of Armour & Company in one cup of hot water. The duodenal contents are examined first in the fasting condition, then one cup of bouillon is given by mouth, and the duodenal contents are aspirated every half-hour for a period of two hours. This is carried out as follows: Give patient the duodenal tube in the evening after a light supper. Note the length of tube which has entered the buccal cavity before retiring. The tube used is that recommended by Einhorn with three marks, Mark I, Mark II, and Mark III. Mark III should have reached the mouth; if not, the patient is given milk or water and the tube in this way made to go in beyond the III mark before going to sleep. Attach the tube, lying outside the mouth, with a piece of adhesive tape to the cheek. By early morning the capsule end of the tube has usually reached the duodenum.

Einhorn states that in normal cases there was an alkalinity of the duodenal contents of 30, while one-half hour after the bouillon test meal it was 10, then it rose to 20, 25 and ultimately to 30. The ferments were strongest in the fasting condition, while they were weakest one-half hour after the test meal, to increase in strength later on. In pathologic cases the degree of alkalinity of the duodenal contents appeared to be dependent to a certain extent upon the state of gastric secretion. Einhorn states² that cases of hyperchlorhydria showed a lowered alkalinity while cases of subacidity manifested an increased alkalinity. Other factors play a role, chief of which are pancreatic and duodenal juices and the bile. He differentiates four types of alkalinity curves: **type I**—decrease of the alkalinity during the first half hour after the bouillon test meal, then a steady increase; **type II**—increase of alkalinity between one-half and one hour after the bouillon, then slight decrease; **type III**—increase of alkalinity during the first half hour after the bouillon, then slight decrease up to one hour, then again increase to one and one-half hours, thereupon again a decrease; **type IV**—at first a steady increase of alkalinity up to one and one-half hours after the bouillon test meal, then an abrupt descent of the curve, so that at two hours after the bouillon the contents are acid instead of alkaline.

Einhorn³ gives the following four types of reaction in peptic ulcers:

1. Duodenal contents are always alkaline in the fasting state as well as at any time after the bouillon test meal.
2. The duodenal contents are alkaline in the fasting state and at times acid after the bouillon test meal.
3. The duodenal contents are acid in the fasting condition and at certain periods after the bouillon test meal.
4. The duodenal contents are always acid in the fasting state as well as at any time after the bouillon test meal.

Type I represents the normal. In a number of peptic ulcers the abnormal types (II, III, and IV) were found. The severity of the disturbance is least in type II, greater in type III, and greatest in type IV.

(B) Bilirubin

Bile pigment is usually present in the duodenal contents in sufficient amount to impart a tinge of yellow to the fluid. The depth of color is a

²Loc. cit.

³Einhorn, Max: J. A. M. A. 77: 1471, 1921.

useful guide to the amount present. Report as +, ++, +++; that is, a small, moderate, or large amount, respectively. If bile is present, the diagnosis of complete obstruction of the hepatic or common bile duct is ruled out. Its absence does not exclude the possibility of bile reaching the duodenum at some time. .

(C) Urobilin

Urobilin is a derivative of bilirubin. It is doubtful whether or not the former idea of its identity with hydrobilirubin is correct. Urobilin is normally found in the duodenal contents just as it is in urine.

When we find an increase of urobilinogen and urobilin in the duodenal contents, it means the same as an increase of these substances in the stool. The examination of feces and duodenal contents for these ingredients indicates just how much blood destruction is going on. In other words, they are extremely important in the diagnosis of the hemolytic anemias. In the study of anemias in general, when possible, the estimation of urobilin is interesting but not necessarily important.

The most satisfactory clinical method for urobilin is that of Wilbur and Addis, which Schneider applies to the duodenal contents as follows:

Technic.—

To 10 c.c. of duodenal contents add 10 c.c. of saturated alcoholic solution of zinc acetate. Shake well and filter.

To 10 c.c. of the filtrate add 1 c.c. of Ehrlich's reagent. (See page 73.)

Mix and let stand in a dark place for fifteen minutes.

Examine with a spectroscope and dilute with 60% alcohol until the bands of both urobilin and urobilinogen have disappeared exactly as is described for urobilin in feces. Calculate the dilution value for 1,000 c.c. of duodenal contents, remembering that the filtrate used represented 5 c.c. of duodenal fluid. If, for example, the urobilin and urobilinogen bands disappeared when the 10 c.c. of filtrate was diluted to 80 c.c. and to 40 c.c., respectively, then the dilution value of 5 c.c. of duodenal fluid is 16 for urobilin and 8 for urobilinogen; and for 1,000 c.c. it would be 200 times this, or 3,200 and 1,600, with a total dilution value of 4,800.

Schneider found that the maximum for healthy medical students is about 1,000 dilutions, and that urobilinogen is never present. In pernicious anemia and hemolytic jaundice urobilinogen is generally present, the total dilution value usually reaching 3,000 to 5,000. Giffin, Sanford, and Szlapka found a striking decrease following splenectomy, particularly in pernicious anemia.

Schneider used these figures in the following formula, which aims to express the relation between blood regeneration and blood destruction in the form of an index number which he designated as the H-H (hematopoietic-hemolytic) Index:

$$\text{hemolytic) Index: } \frac{Z + Y}{6} = \text{H-H index, } Z \text{ representing the total duodenal}$$

urobilin-dilution value in thousands, and Y the red corpuscle count in millions. Under normal conditions (urobilin dilutions 1,000, red cell count 5,000,000) the index is 1. When hemolysis is very active, it will be above 1 unless counterbalanced by very deficient blood formation. When blood regeneration fails the index will be below 1. Such an index is artificial and arbitrary, but is useful in stressing the importance of considering the ratio of blood formation to blood destruction in the course of an anemia.

Microscopic Examination of Duodenal Contents

Unless the duodenal fluid is examined within a few minutes after it is secured, the cellular elements may be damaged or destroyed by the ferments. The method is the same as for fresh urine. Normally only an occasional leukocyte or epithelial cell can be found. Pathologically these may be present in increased numbers, but no definite diagnostic inferences can be drawn. A great excess of pus corpuscles suggests inflammation of the duodenum or biliary tract. *Strongyloides stercoralis* and *Giardia lamblia* have been found, sometimes in great numbers. Cystic and vegetative forms of *Endamoeba histolytica* have also been found, and in such cases infection of the liver or bile passages is inferred.

Bacteriologic Examination.—At the present time very little of clinical value can be learned from a bacteriologic study. Normally the fluid is sterile or contains only a few gram-positive cocci. Bacteria seen in the direct microscopic examination are mostly dead.

For bacteriologic examination the duodenal fluid is obtained in the usual way, with the following precautions to prevent contamination:

Sterilize the tube by boiling. Slip over the metal tip a gelatin capsule which has been soaked in alcohol for several days. Dip the gelatin-covered tip in thin shellac several times, letting it dry after each coating. Introduce the tube in the usual way. When it has entered the duodenum, the gelatin bag may be removed by forcing in a little air or a few cubic centimeters of sterile water. (See pages 1609 ff. and 1627 ff. for examination of gastric contents for tubercle bacilli.)

Cytologic examination is discussed on pages 2192 ff.

The Value of Protein Nitrogen and Nonprotein Nitrogen Determinations on Gastric Juice

A series of papers has been written by Lay Martin¹ on this question.

Normal nonprotein nitrogen is usually found within certain quantitative limits; the fasting juice in normal cases contains more protein and nonprotein nitrogen than the succeeding ones.

Benign achlorhydria, under stimulation with histamine, showed higher values than those of the normal, but lower than those of gastric carcinoma or of nephritis with marked increase in blood nonprotein nitrogen.

Does the stomach act as an agent for removal of these products of metabolism from the body? Are they reabsorbed in the lower intestinal tract? Are they destroyed or benignly conjugated? Williams and Dick² attempted to answer these questions by establishing standards of normality for intestinal excretions of nonprotein nitrogen products after ingestion of magnesium sulphate. They showed they are quantitatively increased in nephritis.

Withdrawal of Contents and Precipitation of Proteins.—A duodenal tube is placed in the dependent portion of the stomach of a patient who has fasted for fifteen hours, the position of the tube being verified by fluoroscopy. Entire fasting secretion is removed and placed in a refrigerator.

¹Martin, L.: Bull. Johns Hopkins Hosp. 49: 286, 1931. J. A. M. A. 100: 1475, 1933.

²Williams, J. L., and Dick, G. F.: J. A. M. A. 100: 484, 1933.

Gastric juice flow is stimulated by hypodermic injection of histamine (ergamine acid phosphate) in amounts equivalent to 0.005 mg. per kilogram of body weight. Entire flow of gastric juice is collected by continuous siphonage into a narrow container surrounded by ice. All specimens with bile tinge are discarded. After gastric flow stops, all gastric juice is filtered in a refrigerator at 7° C., giving a limpid fluid free of mucus.

Proteins of the gastric juice are precipitated by tungstic acid at pH 3.5. Therefore a protein-free filtrate is obtainable which can be used by methods described for the estimation of amino acid, urea, ammonia and nonprotein nitrogen. Total nitrogen for the whole mixture is determined by the Kjeldahl method. The precipitate is redissolved in alkali and the protein nitrogen determined by the same technic.

Normal Averages.—Protein of the mucus-free gastric juice averaged 22.59 mg. of nitrogen per 100 c.c. of gastric juice.

The nonprotein nitrogen was 25.5 mg. per 100 c.c. of gastric juice.

Amino acid nitrogen averaged 7.22 mg. of nitrogen per 100 c.c. of gastric juice.

Urea averaged 2.58 mg. of nitrogen per 100 c.c. of gastric juice.

Ammonia nitrogen averaged 5.19 mg. of nitrogen per 100 c.c. of gastric juice, far more than the negligible amount in blood, suggesting speculation concerning the purpose of the urea-splitting enzyme appearing in the gastric juice and the gastric mucosa.

Uric acid averaged 1.5 mg. per 100 c.c. of gastric juice.

Accordingly, there are 8 mg. of nonprotein nitrogen unaccounted for.

Results in Different Cases.—In benign achlorhydria, protein and nonprotein nitrogen double that of the control group. Compared with seven cases of pernicious anemia, it was found that there was much more amino acid nitrogen and urea nitrogen in pernicious anemia than in benign achlorhydria with consequent rise in nonprotein nitrogen.

In proved cases of carcinoma of the stomach, protein and nonprotein nitrogen fractions increased from four to six times the normal.

Summary:

1. Normal gastric juice shows rather constant figures for protein and nonprotein nitrogen fractions (amino acid, urea, uric acid, and ammonia).
2. In benign achlorhydrias the amounts increased twofold.
3. In pernicious anemia the same increase was found plus still greater increase in amounts of amino acid and urea.
4. In carcinoma of the stomach with achlorhydria, very large amounts of protein and nonprotein nitrogen were found.

Methods for Determination.¹—

Total nitrogen has been determined for normal gastric juice by the Kjeldahl method.

To obtain a protein-free filtrate, it was necessary to devise a method for the precipitation of these materials from a medium with a normal hydrogen ion concentration around 2; in the achylia it was around 7. Blood proteins

¹Martin, Lay: Bull. Johns Hopkins Hosp. 44: 286, 1931.

are precipitated by tungstic acid according to the method of Folin and Wu at a pH of about 7. This method, when applied to gastric juice, in most cases gives no precipitate. Only when the hydrogen ion concentration has been increased to a pH above 4 is one able to precipitate the maximum amount of protein. Routinely this was done in the following manner:

Technic.—

To gastric juice add one-half the amount of 10% sodium tungstate. As this solution is gradually added, a cloudy precipitate is formed which is reabsorbed as the pH decreases. Two-thirds normal sulphuric acid is then added, drop by drop, until the pH of the solution is brought to about 3.5. From many trials it has been found that this is about the optimal point of the heaviest precipitation. This end point can be recognized by means of Congo red paper which just begins to turn from light to dark blue. The material is then made to any required dilution, usually about 4 to 1. In the achylia, experience has shown that a 6 to 1 dilution is sufficient. Filtration through a Whatman No. 42 filter paper will usually produce a perfectly clear filtrate. If this does not appear at first, refiltering through the same paper will usually bring about the desired result. Standing overnight in a refrigerator will also increase the size of the precipitate particles and make filtration easier.

Biuret Tests.—

Biuret tests done upon filtrates are negative or show so little color that one feels sure that the amount of protein material can have no significant influence on the findings. A comparison of the biuret reaction on the unfiltered and the filtered portion is sufficient to justify the conclusion that, even when this very faint trace of lavender or pink is seen, the amount of protein therein is certainly negligible.

Nonprotein Nitrogen.—The estimation of the total nitrogen on the filtrate is carried out by the usual Folin and Wu nesslerization method. It lies normally between 20 and 48 mg. per cent. In simple achylia, the percentage is higher, up to 30 and 90 mg. per cent. In achylia complicated by carcinoma the figures have been as high as 60 to 150 mg. per cent.

Amino Acids.—The amount of amino acids was determined by the blood filtrate method of Folin. Due to the higher acidity, much more sodium carbonate must be used before the phenolphthalein will impart a permanent pink color to the solution.

Urea.—Urea is determined by first estimating the amount of free ammonia plus the amount of ammonia liberated by the action of urease; from this result subtract the predetermined amount of ammonia.

Ammonia.—Ammonia is determined by two methods: first, unfiltered gastric juice, usually 5 c.c., by the microaeration method of Folin and MacCallum; second, upon the filtrate using the method of Folin and Wu.

Determination of Blood Group by Examination of Gastric Contents. See agglutinin inhibitory tests, Chapter V, pages 1169 ff.

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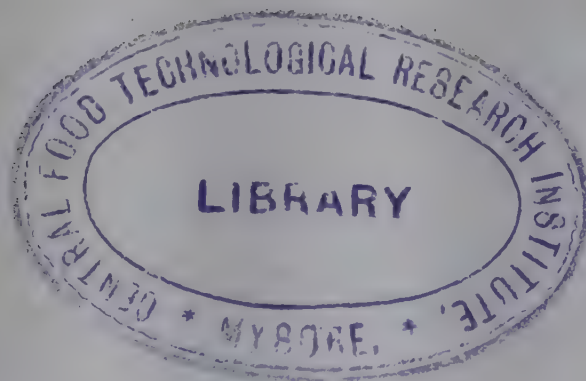
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